

## PCT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

WALTON, Sean, M.  
Mewburn Ellis  
York House  
23 Kingsway  
London WC2B 6HP  
ROYAUME-UNI

Date of mailing (day/month/year) 06 July 1999 (06.07.99)	<b>IMPORTANT NOTIFICATION</b>
Applicant's or agent's file reference SMW/BP5710520	
International application No. PCT/GB98/02115	International filing date (day/month/year) 16 July 1998 (16.07.98)

## 1. The following indications appeared on record concerning:

☒ the applicant
                 
 ☐ the inventor
                 
 ☐ the agent
                 
 ☐ the common representative

## Name and Address

CANCER RESEARCH CAMPAIGN  
TECHNOLOGY LIMITED  
Cambridge House  
6-10 Cambridge Terrace  
Regent Park  
London NW1 4JL  
United Kingdom

## State of Nationality

GB

## State of Residence

GB

Telephone No.

Facsimile No.

Teleprinter No.

## 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☒ the person
                 
 ☒ the name
                 
 ☒ the address
                 
 ☐ the nationality
                 
 ☐ the residence

## Name and Address

KuDOS PHARMACEUTICALS LIMITED  
327-329 Cambridge Science Park  
Cambridge CB4 4GW  
United Kingdom

## State of Nationality

GB

## State of Residence

GB

Telephone No.

Facsimile No.

Teleprinter No.

## 3. Further observations, if necessary:

## 4. A copy of this notification has been sent to:

<input checked="" type="checkbox"/> the receiving Office	<input type="checkbox"/> the designated Offices concerned
<input type="checkbox"/> the International Searching Authority	<input checked="" type="checkbox"/> the elected Offices concerned
<input checked="" type="checkbox"/> the International Preliminary Examining Authority	<input type="checkbox"/> other:

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland  Facsimile No.: (41-22) 740.14.35	Authorized officer  Mougamadou Abidine  Telephone No.: (41-22) 338.83.38
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## PCT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING  
OF A CHANGE(PCT Rule 92bis.1 and  
Administrative Instructions, Section 422)

From the INTERNATIONAL BUREAU

To:

WALTON, Sean, M.  
Mewburn Ellis  
York House  
23 Kingsway  
London WC2B 6HP  
ROYAUME-UNI

<b>Date of mailing</b> (day/month/year) 22 June 1999 (22.06.99)	<b>IMPORTANT NOTIFICATION</b>
<b>Applicant's or agent's file reference</b> SMW/BP5710520	
<b>International application No.</b> PCT/GB98/02115	<b>International filing date</b> (day/month/year) 16 July 1998 (16.07.98)

## 1. The following indications appeared on record concerning:

☒ the applicant
 ☒ the inventor
 ☐ the agent
 ☐ the common representative

Name and Address

State of Nationality

State of Residence

Telephone No.

Facsimile No.

Teleprinter No.

## 2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

☐ the person
 ☐ the name
 ☐ the address
 ☐ the nationality
 ☐ the residence

Name and Address

HANN, Byron  
213 Hillside Avenue  
Piedmont, CA 94611  
United States of America

State of Nationality

US

State of Residence

US

Telephone No.

Facsimile No.

Teleprinter No.

## 3. Further observations, if necessary:

**The above-mentioned applicant/inventor should be deleted on the records.**

## 4. A copy of this notification has been sent to:

☒ the receiving Office
 ☐ the designated Offices concerned  
☐ the International Searching Authority
 ☒ the elected Offices concerned  
☒ the International Preliminary Examining Authority
 ☐ other:
The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Mougamadou Abidine

Telephone No.: (41-22) 338.83.38



## PATENT COOPERATION TREATY

PCT

## NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

United States Patent and Trademark  
Office  
(Box PCT)  
Crystal Plaza 2  
Washington, DC 20231  
ÉTATS-UNIS D'AMÉRIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 03 March 1999 (03.03.99)	
International application No. PCT/GB98/02115	Applicant's or agent's file reference SMW/BP5710520
International filing date (day/month/year) 16 July 1998 (16.07.98)	Priority date (day/month/year) 16 July 1997 (16.07.97)
Applicant LANE, David, Phillip et al	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:

11 February 1999 (11.02.99)

☐ in a notice effecting later election filed with the International Bureau on:2. The election ☒ was☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer Nicola Wolff
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38



# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>SMW/BP5710520</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/GB 98/ 02115</b>	International filing date (day/month/year) <b>16/07/1998</b>	(Earliest) Priority Date (day/month/year) <b>16/07/1997</b>
Applicant <b>CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 7 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

### 1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☒ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☒ Certain claims were found unsearchable (See Box I).

3. ☒ Unity of invention is lacking (see Box II).

4. With regard to the title,

☐ the text is approved as submitted by the applicant.

☒ the text has been established by this Authority to read as follows:

**INTERACTIONS OF ATM, ATR OR DNA-PK WITH P53**

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawings to be published with the abstract is Figure No.

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☒ None of the figures.





# INTERNATIONAL SEARCH REPORT

national application No.  
PCT/GB 98/02115

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☒ Claims Nos.: **5,6 (PARTLY) AND 21-23 (ENTIRELY)**  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
**see FURTHER INFORMATION sheet PCT/ISA/210**
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

**see additional sheet**

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.



FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: 5,6 (PARTLY) AND 21-23 (ENTIRELY)

The subject-matter of claims 5 and 6 in their broadest scope is not defined by technical features that allow the formulation of a meaningful search. These claims do not meet the requirements of Article 6 and Rule 6.3(a) PCT to the extent that a meaningful search is not possible in the entire scope claimed. The subject of the search for the agents of claims 5 and 6 has been limited to the subject-matter of claims 7-16.

As it lies not within the ISA's competence to test a compounds for their capabilities of modulating interactions the scope of the search was formulated by the structural features only.

The subject-matter of claims 21-23 is not defined by technical features that allow the formulation of a meaningful search. These claims do not meet the requirements of Article 6 and Rule 6.3(a) PCT to the extent that a meaningful search is not possible



**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-18,28,29

Methods for identifying compounds able to modulate interaction between p53 and DNA binding kinases having the PI3 kinase domain, in particular by phosphorylation of the N-terminus of p53 (ser15) by said kinases, agents so identifiable and medical use of such agents.

2. Claims: 19-27

Methods for identifying compounds able to affect DNA binding by DNA binding kinases having the PI3 kinase domain, agents so identifiable, medical use of such agents and methods involving such binding for purifying ATM or ATR.



09/462 962

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification<sup>6</sup> :</b> <b>G01N 33/574, 33/68, C12Q 1/48</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 99/04266</b> <b>(43) International Publication Date:</b> 28 January 1999 (28.01.99)
<b>(21) International Application Number:</b> PCT/GB98/02115 <b>(22) International Filing Date:</b> 16 July 1998 (16.07.98) <b>(30) Priority Data:</b> 9714971.0      16 July 1997 (16.07.97)      GB <b>(71) Applicant (for all designated States except US):</b> CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB]; Cambridge House, 6-10 Cambridge Terrace, Regent Park, London NW1 4JL (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LANE, David, Phillip [GB/GB]; Magicwell House, Quarry Road, Balmullo, Fife KY16 0AN (GB). HANN, Byron [US/US]; 213 Hillside Avenue, Piedmont, CA 94611 (US). JACKSON, Stephen, Philip [GB/GB]; 45 Thornton Road, Girton, Cambridge CB3 0NP (GB). LAKIN, Nicholas, David [GB/GB]; 27 Hobarts Road, Cambridge CB1 3PU (GB). SMITH, Graeme, Cameron, Murray [GB/GB]; 29 The Elms, Milton, Cambridge CB4 2ZQ (GB). <b>(74) Agents:</b> WALTON, Sean, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> <b>(88) Date of publication of the international search report:</b> 19 August 1999 (19.08.99)
<b>(54) Title:</b> INTERACTIONS OF ATM, ATR OR DNA-PK WITH P53 <b>(57) Abstract</b> <p>The interaction of ATM and related protein kinases such as ATR and DNA-PK with p53 is disclosed, in particular the phosphorylation of Ser15 and Thr18 by these proteins. The activity of the proteins is shown to increase in the presence of DNA. Assays for modulators of phosphorylation by the interaction between the proteins and p53 or other proteins having similar phosphorylation sites are provided. Methods of purifying ATM or ATR employing DNA or NTA are also disclosed.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
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CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/02115

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 G01N33/574 G01N33/68 C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N C12Q C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>M.S. MEYN: "Ataxia-telangiectasia and cellular responses to DNA damage"  CANCER RESEARCH,  vol. 55, 15 December 1995, pages  5991-6001, XP002095208  cited in the application  see the whole document  see page 5998, left-hand column, line  28-38</p> <p style="text-align: center;">--- -/--</p>	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&amp;" document member of the same patent family

Date of the actual completion of the international search

11 May 1999

Date of mailing of the international search report

21.06.1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Hoekstra, S



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02115

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SAVITSKY K ET AL: "A SINGLE ATAXIA TELANGIECTASIA GENE WITH A PRODUCT SIMILAR TO PI-3 KINASE" SCIENCE, vol. 268, 23 June 1996, pages 1749-1753, XP002061971 cited in the application see the whole document see figure 3 ---	5,6, 13-16,28
X	SAVITSKY K ET AL: "THE COMPLETE SEQUENCE OF THE CODING REGION OF THE ATM GENE REVEALS SIMILARITY TO CELL CYCLE REGULATORS IN DIFFERENT SPECIES" HUMAN MOLECULAR GENETICS, vol. 4, no. 11, 1995, pages 2025-2032, XP002061974 cited in the application see the whole document see figure 4 ---	5,6, 13-16,28
Y	see figure 2	19,20, 24,25
X	CIMPRICH K A ET AL: "CDNA CLONING AND GENE MAPPING OF A CANDIDATE HUMAN CELL CYCLE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, April 1996, pages 2850-2855, XP002023632 cited in the application see the whole document see figure 2 ---	29
P,X	SHIEH, S-Y.: "DNA Damage-induced phosphorylation of p53 alleviates inhibition by MDM2" CELL, vol. 91, 31 October 1997, pages 325-334, XP002095209 see the whole document see page 331, right-hand column, line 27-35 ---	1-18
E	WO 98 56391 A (HARVARD COLLEGE ;LEDER PHILIP (US); WESTPHAL CHRISTOPH H (US)) 17 December 1998 see page 12 ---	13-16
A	WO 94 12202 A (UNIV DUNDEE ;LANE DAVID PHILIP (GB); HUPP THEODORE ROBERT (GB)) 9 June 1994 see the whole document ---	1-18
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	-/--	



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 98/02115

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HARTLEY K O ET AL: "DNA-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT: A RELATIVE OF PHOSPHATIDYLINOSITOL 3-KINASE AND THE ATAXIA TELANGIECTASIA GENE PRODUCT" CELL, vol. 82, 8 September 1995, pages 849-856, XP002065036 cited in the application see the whole document ---	1-18
A	ENOCH, T. AND NORBURY, C.: " Cellular responses to DNA damage: cell-cycle checkpoints, apoptosis and the roles of p53 and ATM." TIBS, vol. 20, October 1995, pages 426-430, XP002095210 see the whole document ---	1-18
Y	A. SUWA, ET AL.: "DNA dependent protein kinase (Ku protein-p350 complex) assembles on double stranded DNA" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, July 1994, pages 6904-6908, XP002102470 WASHINGTON US see abstract see page 6905, right-hand column, line 20 - page 6907, left-hand column, line 19 ---	19,20, 24,25
A	WO 97 18323 A (ICOS CORP ;HOEKSTRA MERL F (US); HOLTZMAN DOUG A (US); KEEGAN KATH) 22 May 1997 see abstract -----	19,20, 24-27



# INTERNATIONAL SEARCH REPORT

Info on patent family members

International Application No

PCT/JP 98/02115

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9856391	A	17-12-1998	AU	8068198 A	30-12-1998
WO 9412202	A	09-06-1994	AU	680216 B	24-07-1997
			AU	5533194 A	22-06-1994
			CA	2150265 A	09-06-1994
			EP	0675729 A	11-10-1995
			JP	8505607 T	18-06-1996
WO 9718323	A	22-05-1997	AU	1461197 A	05-06-1997
			CA	2210650 A	22-05-1997
			CN	1199420 A	18-11-1998
			CZ	9702547 A	18-03-1998
			EP	0807169 A	19-11-1997
			FI	973005 A	15-09-1997
			NO	973279 A	16-09-1997
			PL	322876 A	02-03-1998
			SK	111597 A	06-05-1998





INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference SMW/BP5710520		See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) <b>FOR FURTHER ACTION</b>
International application No. PCT/GB98/02115	International filing date (day/month/year) 16/07/1998	Priority date (day/month/year) 16/07/1997
International Patent Classification (IPC) or national classification and IPC G01N33/68		
Applicant KUDOS Pharmaceuticals LIMITED et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 9 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand  11/02/1999	Date of completion of this report  10. 11. 99
Name and mailing address of the international preliminary examining authority:   European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized officer  Hoekstra, S  Telephone No. +31 70 340 2847  



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB98/02115

**I. Basis of the report**

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

**Description, pages:**

1-82 as originally filed

**Claims, No.:**

1-27 as received on 16/09/1999 with letter of 15/09/1999

**Drawings, sheets:**

1/32-32/32 as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:  
☐ the claims, Nos.:  
☐ the drawings, sheets:

3. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

**see separate sheet**

4. Additional observations, if necessary:

**III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.  
☒ claims Nos. 5,6 (partly).

because:



# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB98/02115

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):
- ☒ the claims, or said claims Nos. 5,6 are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .

## IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

- ☐ restricted the claims.
- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☒ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

- ☐ complied with.
- ☒ not complied with for the following reasons:

**see separate sheet**

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

- ☐ all parts.
- ☒ the parts relating to claims Nos. 1-16, 26,27.



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/GB98/02115

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	1-4,11-14
	No:	Claims	7-10,15,16,26,27
Inventive step (IS)	Yes:	Claims	2
	No:	Claims	1,3,4,7-16, 26, 27
Industrial applicability (IA)	Yes:	Claims	1-4,7-16, 26,27
	No:	Claims	

**2. Citations and explanations**

**see separate sheet**

**VI. Certain documents cited**

**1. Certain published documents (Rule 70.10)**

and / or

**2. Non-written disclosures (Rule 70.9)**

**see separate sheet**

**VII. Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:

**see separate sheet**





**Re Item I**

**Basis of the opinion**

The limitation of of the peptides by size and in particular by the arbitrary boundary of 40 amino acids is a novel teaching going beyond the disclosure of the international application as filed (Article 34(2)(b) PCT). The preliminary examination report shall be made up as if this amendments had not been made (Rule 70.2(c) PCT).

**Re Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

The subject-matter of claims 5 and 6 in their broadest scope does not define the subject-matter for which protection is sought because no technical features that allow a distinction over the prior art are present in these claims which hence do not meet the requirements of Article 6 and Rule 6.3(a) PCT to the extent that a meaningful preliminary examination is not possible. As it lies not within the ISA's competence to test compounds for their capabilities of modulating interactions the preliminary examination is carried out having in mind the structural features of claims 7-16.

**Re Item IV**

**Lack of unity of invention**

The International Preliminary Examination Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-16,26,27

Methods for identifying compounds able to modulate interaction between p53 and DNA binding kinases having the PI3 kinase domain, in particular by phosphorylation of the N-terminus of p53 (ser15) by said kinases, agents so identifiable and medical use of such agents.



2. Claims: 17-25

Methods for identifying compounds able to affect DNA binding by DNA binding kinases having the PI3 kinase domain, agents so identifiable, medical use of such agents and methods involving such binding for purifying ATM or ATR.

The only common feature that is present in all claimed inventions is the involvement of ATM, DNA-PK or ATR, or a protein having an associated kinase activity which is, in view of the description and the prior art at hand, considered as a class of kinases defined as DNA binding kinases having the PI3 kinase domain and which are all involved in signalling pathways relating to DNA repair.

The problem to be solved is to provide agents that may affect the biochemistry of the signal transduction which is impaired in A-T patients. The underlying technical problem to be solved is, as is derivable from the description, to elucidate the biochemical role of ATM which is per se known in the art as involved in signal transduction relating to DNA repair (M.S. Meyn, 1995, Cancer Research, vol. 55, pp. 5991-6001; designated hereinafter as D1)

One solution presented in the application relates to the discovery that the above identified and widely recognised class of DNA-binding kinases all phosphorylate p53 (in particular at ser15).

This leads to the formulation of assays for compounds involving determining the compound's effect on interactions of ATM or its relatives ATR and DNA-PK with p53.

All inventions technically linked to this discovery and characterised by the technical feature of phosphorylation of p53 are considered to share a general inventive concept (Subject 1).



It is however considered that the claimed inventions relating to the DNA-binding properties (Subject 2) of this kinase family is not characterised by the technical feature of the shared p53 substrate, neither as same nor as corresponding technical feature. Vice versa the claimed inventions involving interaction with p53 do not involve DNA-binding as a technical feature.

Because the application lacks a feature that defines a contribution which each of the claimed inventions, considered as a whole, makes over the prior art i.e. one special technical feature in the sense of Rule 13.2, it is concluded that the application does not relate to a single general inventive concept as required by Rule 13.1 PCT. The application does hence not meet the requirement of Article 3(4)(iii) PCT.

**Re Item V**

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The present application does not meet the requirements of Article 33(2)(3) PCT because the subject-matter of claims 7-10,15,16,26,27 is not novel whereas the subject-matter of claims 1,3,4 and 11-14 is considered not inventive.
2. Both Savitsky et al. disclosures disclosed the ATM gene. The polypeptide encoded thereby and the various fragments thereof are implicitly disclosed therein and made available to the public thereby prejudicing the novelty of claim 26 and claims relating to functionally defined fragments thereof.
3. Cimprich et al. disclosed the sequence of the FRP1 polypeptide (fig. 2) which is identical to the ATR gene product (see application page 6, lines 28-30), the subject-matter of claim 27 has thus been made available to the public.
4. Claims 7-10 relate to fragments of the well-known p53 protein. It cannot be



maintained that p53 per se or fragments thereof which are not structurally defined could form a contribution to the state of the art.

5. Meyn et al. speculated on the relation between ATM and p53 (See "Discussion and predictions") which is confirmed by the present invention.

No evidence, however, is presented by Meyn et al. supporting his postulation, which logically followed from the DNA damage surveillance network model, that ATM (or ATR) phosphorylates p53, in analogy to other PI3-kinase containing protein phosphorylases.

6. It is however, clearly derivable from Meyn et al. that it is known in the art that from the other related PI3 kinase domain carrying polypeptides (to be considered as the proteins having associated kinase activity appearing as alternatives to ATM in the claims) at least DNA-PK indeed does phosphorylate p53 (p. 5997, right column, lines 8 and 9 (See also Savitsky in Human Molecular Genetics vol 4(11) p. 2030, right column, lines 27-31).
7. Thus although this information is new for the relation between ATM (and ATR) and p53, the assays of claims 1, 3 and 4, follows in an obvious manner from at least Meyn et al. taken alone in view of the conclusion drawn by Meyn et al that ATM may play its biological role by "...physically interacting with the p53 protein..." (Page 5998, left column, lines 36 - 40).
8. Any functionally relevant fragment of the involved molecules (ATM, p53 etc.) also follows in an obvious manner from Meyn et al. in combination with any disclosure of the complete pure molecules.
9. The contribution to the state of the art made by the present inventors lies in the discovery of p53 being a phosphorylation substrate of ATM and ATR which is reflected correctly in claim 2. Subject-matter which is technically limited to ATM and its relation to phosphorylation of p53 is considered to meet the requirements of Article 33(2) and (3) PCT.





**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/GB98/02115

**Re Item VI**

1. Certain published documents (Rule 70.10)

WO9856391

PUB: 17.12.98

Fil:11.06.98

PRIO:13.06.97

**Re Item VII**

**Certain defects in the international application:**

The scope of claims 5-16 extends to compounds only defined by their ability to be identified by the methods of claim 1-4. It is considered that the wide scope of these claims is not sufficiently supported by the technical disclosure of the invention in the description. These claims do therefor not meet the requirements of Article 5, 6 and Rules 5.1(a)(iii) and 6(3)a PCT. Only claims limited to structurally defined fragments could be considered as meeting the requirements of Article 5, 6 and Rules 5.1(a)(iii) and 6(3)a PCT.



**Claims:**

1. An assay method for a compound able to modulate the interaction between (i) ATM or ATR and (ii) p53, the method including the steps of:

5 (a) bringing into contact a substance including a peptide fragment of (i) or a derivative, variant or analogue thereof, a substance including the relevant fragment of p53, or a variant, derivative or analogue thereof, and a test compound; and

10 (b) determining interaction or binding between said substances and test compound.

2. An assay method for a compound able to modulate the interaction between (i) ATM or ATR and (ii) p53, the method including the steps of:

15 (a) bringing into contact a substance which includes at least a fragment of (i) which phosphorylates p53, a substance which includes at least a fragment of p53, which includes a site which is phosphorylated by (i), and a test compound; and

20 (b) determining phosphorylation at said site.

3. An assay method for a compound able to affect p53 activity by modulation of interaction between (i) ATM or ATR and (ii) p53, the method including the steps of:

25 (a) bringing into contact a substance which is p53 or a fragment, derivative, variant or analogue thereof, and a test compound; and

30 (b) determining p53 activity in the presence and absence of (i).

4. The method according to any one of claims 1 to 3 wherein said compound is able to modulate interaction between ATM and p53.

35 5. An agent capable of modulating interaction between (i) ATM or ATR and (ii) p53 obtained using a method

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according to any of claims 1 to 4.

5 6. An agent according to claim 5 capable of modulating ATM-mediated phosphorylation on p53 or said homologous sites.

10 7. A peptide fragment of p53 of about 40 amino acids or less and capable of modulating interaction between ATM or ATR and p53.

8. A peptide according to claim 7 capable of modulating phosphorylation of p53 by ATM or ATR.

15 9. A peptide according to claim 7 or claim 8 capable of modulating interaction between ATM and p53.

10. A nucleic acid isolate encoding a peptide according to any of claims 7 to 9.

20 11. A peptide fragment of ATM or ATR of about 40 amino acids or less and which is capable of modulating interaction between ATM or ATR and p53.

25 12. A peptide according to claim 11 capable of modulating phosphorylation of p53 by ATM or ATR.

13. The peptide of claim 11 or claim 12 capable of modulating interaction between ATM and p53.

30 14. A nucleic acid isolate encoding a peptide according to any one of claims 11 to 13.

35 15. An agent or peptide fragment or nucleic acid isolate according to any of claims 5 to 14 for use in a method of treatment by therapy involving modulating ATM action.

16. Use of an agent or peptide fragment or nucleic acid



absence of (i).

4. The method according to any one of claims 1 to 3 wherein the protein having an associated kinase activity is DNA-PK or ATR.

5. An agent capable of modulating interaction between (i) ATM or a protein having an associated kinase activity and (ii) p53 or any protein having ATM phosphorylation sites homologous to p53 obtained using a method according to any of claims 1 to 4.

6. An agent according to claim 5 capable of modulating ATM-mediated phosphorylation on p53 or said homologous sites.

7. A peptide fragment of p53 capable of modulating interaction between ATM or a protein having an associated kinase activity and p53.

8. A peptide according to claim 7 capable of modulating phosphorylation of p53 by ATM or by a protein having an associated kinase activity.

9. A peptide according to claim 7 or claim 8 wherein the protein having an associated kinase activity is DNA-PK or ATR.

10. A nucleic acid isolate encoding a peptide according to any of claims 7 to 9.

11. A peptide fragment of ATM or a protein having an associated kinase activity which is capable of modulating interaction between ATM or the protein and p53.

12. A peptide according to claim 11 capable of modulating phosphorylation of p53 by ATM or a protein

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having an associated kinase activity.

13. The peptide of claim 11 or claim 12 wherein the protein having an associated kinase activity is DNA-PK or ATR.

14. A nucleic acid isolate encoding a peptide according to any one of claims 11 to 13.

15. An agent or peptide fragment or nucleic acid isolate according to any of claims 5 to 14 for use in a method of treatment by therapy involving modulating ATM action.

16. Use of an agent or peptide fragment or nucleic acid isolate according to any of claims 5 to 15 in the manufacture of a medicament for modulating ATM action.

17. An assay method for a compound able to affect DNA binding by ATM or a protein having an associated kinase activity, the method including the steps of:

(a) bringing into contact a substance which is ATM or a protein having an associated kinase activity, or a fragment, variant or derivative which is able to bind DNA, DNA, and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of DNA binding by ATM or the protein having an associated kinase activity, said substance binds DNA; and

(b) determining binding between said substance and said DNA.

18. The assay method according to claim 17 wherein the protein having an associated kinase activity is DNA-PK or ATR.

19. An agent capable of affecting DNA binding by ATM obtained using a method according to claim 17 or claim 18.



20. An agent according to claim 19 for use in a method of treatment by therapy involving modulating ATM action.

5 21. Use of an agent according to claim 19 in the manufacture of a medicament for modulating ATM action.

10 22. A method of purifying ATM or ATR including contacting a mixture of molecules including ATM or ATR with DNA, washing off molecules which do not bind the DNA, and recovering ATM or ATR from the DNA-bound fraction.

23. Use of DNA for purifying ATM or ATR.

15 24. A method of purifying ATM or ATR, including contacting a mixture of molecules including ATM or ATR with NTA, washing off molecules which do not bind the NTA, and recovering ATM or ATR from the NTA-bound fraction.

20 25. A method according to claim 24 wherein the mixture is contacted with NTA in the presence of  $\text{Ni}^{2+}$ .

25 26. Substantially pure ATM.

27. Substantially pure ATR.

30



with NTA, washing off molecules which do not bind the NTA, and recovering ATM or ATR from the NTA-bound fraction.

- 5      25. A method according to claim 24 wherein the mixture is contacted with NTA in the presence of  $\text{Ni}^{2+}$ .
26. Substantially pure ATM.
- 10     27. Substantially pure ATR.



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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/GB98/02115 <b>(22) International Filing Date:</b> 16 July 1998 (16.07.98) <b>(30) Priority Data:</b> 9714971.0                      16 July 1997 (16.07.97)                      GB <b>(71) Applicant (for all designated States except US):</b> CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB]; Cambridge House, 6-10 Cambridge Terrace, Regent Park, London NW1 4JL (GB). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LANE, David, Phillip [GB/GB]; Magicwell House, Quarry Road, Balmullo, Fife KY16 0AN (GB). HANN, Byron [US/US]; 213 Hillside Avenue, Piedmont, CA 94611 (US). JACKSON, Stephen, Philip [GB/GB]; 45 Thornton Road, Girton, Cambridge CB3 0NP (GB). LAKIN, Nicholas, David [GB/GB]; 27 Hobarts Road, Cambridge CB1 3PU (GB). SMITH, Graeme, Cameron, Murray [GB/GB]; 29 The Elms, Milton, Cambridge CB4 2ZQ (GB). <b>(74) Agents:</b> WALTON, Sean, M. et al.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> ASSAYS, THERAPEUTIC METHODS AND MEANS		
<b>(57) Abstract</b>  The interaction of ATM and related protein kinases such as ATR and DNA-PK with p53 is disclosed, in particular the phosphorylation of Ser15 and Thr18 by these proteins. The activity of the proteins is shown to increase in the presence of DNA. Assays for modulators of phosphorylation by the interaction between the proteins and p53 or other proteins having similar phosphorylation sites are provided. Methods of purifying ATM or ATR employing DNA or NTA are also disclosed.		

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**ASSAYS, THERAPEUTIC METHODS AND MEANS**

The present invention relates to screening methods, peptides, mimetics, and methods of use based on the surprising discovery and characterisation of an interaction between known proteins, and thus numerous cellular processes of interest in therapeutic contexts. The proteins in question are ATM and p53, and the inventors have found that ATM phosphorylates p53 at a number of specific sites. This interaction is observed with other related proteins with associated kinase activity, in particular ATR and DNA-PK, and other proteins having similar phosphorylation sites to p53. Further aspects of the present invention are founded on the discovery that ATM binds DNA and that such binding has an effect on phosphorylation of p53 by ATM.

Ataxia-telangiectasia (A-T) is a human autosomal recessive disorder characterised by a number of debilitating symptoms, including a progressive cerebellar degeneration, oculocutaneous telangiectasia, growth retardation, immune deficiencies and certain characteristics of premature ageing (reviewed in Jackson, 1995; Meyn, 1995; Shiloh, 1995). A-T patients exhibit an approximately 100-fold increased incidence of cancer, with patients being particularly predisposed to malignancies of lymphoid origin. Furthermore, A-T heterozygotes, which comprise ~1% of the population, are reported to exhibit a higher incidence of breast cancer (Easton, 1994; Meyn, 1995), although this remains controversial (Fitzgerald et al., 1997). At the cellular level, A-T is characterised by a high degree of chromosomal instability, radioresistant DNA synthesis, and hypersensitivity to ionising radiation (IR) and radiomimetic drugs. In addition, A-T cells are defective in the radiation induced G1-S, S, and G2-M cell cycle checkpoints that are thought to arrest the cell cycle in response to DNA damage in order to allow repair of the

genome prior to DNA replication or mitosis (Halazonetis et al., 1993; Beamish et al., 1994; Beamish and Lavin, 1994; Khanna et al., 1995; Barlow et al., 1996; Xu and Baltimore, 1996). A-T cells exhibit deficient or  
5 severely delayed induction of p53 in response to IR (Kastan et al., 1992; Khanna and Lavin, 1993; Lu and Lane, 1993; Xu and Baltimore, 1996). p53 mediated transcriptional activation of p21/WAF1/CIP1 and Gadd45, and the subsequent inhibition of G1 cyclin-dependent  
10 kinases, are also defective in A-T cells following IR exposure (Artuso et al., 1995; Khanna et al., 1995). Lu and Lane, 1993, however, reported very little difference in the p53 response from normal and A-T cells.

15 Furthermore, yeast have an ATM homologue (Mec1) but do not have p53 (Goffeau et al.). The best data for a possible substrate for Mec1p is Spk1/Rad53 (Sun et al; Sanchez et al.)

20 The gene mutated in A-T patients, termed ATM (A-T mutated), has been mapped and its cDNA cloned (Savitsky et al., 1995a; Savitsky et al., 1995b). Sequence analyses reveal that the ATM gene encodes a ~350 kDa polypeptide that is a member of the phosphatidylinositol  
25 (PI) 3-kinase family of proteins by virtue of a putative kinase domain in its carboxyl-terminal region (Savitsky et al., 1995a; Savitsky et al., 1995b). Classical PI 3-kinases, such as PI 3-kinase itself, are involved in signal transduction and phosphorylate inositol lipids  
30 that act as intracellular second messengers (reviewed in Kapeller and Cantley, 1994). ATM bears sequence similarity with a subset of the PI 3-kinase protein family that comprises proteins which, like ATM, are involved in cell cycle control and/or in the detection  
35 and signalling of DNA damage (for reviews see Hunter, 1995; Keith and Schreiber, 1995; Zakian, 1995; Jackson, 1996). Included in this sub-group are Saccharomyces

cerevisiae Tor1p and Tor2p and their mammalian homologue FRAP, which control progression into S-phase and, at least in part, function by regulating translation (Brown and Schreiber, 1996). Also in this sub-group is the DNA dependent protein kinase (DNA-PK) catalytic subunit (DNA-PKcs), defects in which lead to sensitivity to IR and an inability to perform site-specific V(D)J recombination (reviewed in Jackson and Jeggo, 1995; Jackson, 1996). Other members of the ATM sub-group of the PI 3-kinase family that have been identified include *S. cerevisiae* Tellp and Mec1p, together with the Mec1p homologues of *Schizosaccharomyces pombe* (rad3), *Drosophila melanogaster* (mei-41) and humans FRP1/ATR; (Keith and Schreiber, 1995; Zakian, 1995; Jackson, 1996). As with ATM, defects in these proteins lead to genomic instability, hypersensitivity towards DNA damaging agents and defects in DNA damage-induced cell cycle checkpoint controls.

ATM is most similar to *S. cerevisiae* Tellp, which has not been shown to have any biochemical function so far (identity and similarity are 45% and 66%, respectively). ATM is much further diverged from DNA-PKcs (28% identical and 51% similar), with essentially the same homology to PI 3-kinase (a *bona fide* lipid kinase: 24% identical and 51% similar). Thus, from the sequence comparisons alone, one could not predict that ATM would be a protein kinase akin to DNA-PKcs or a lipid kinase akin to PI 3-kinase.

Although genetic data indicate an involvement of ATM-like proteins in DNA damage recognition and its repair, the mechanisms by which these proteins function are not well understood. Much is known about the clinical symptoms and cellular phenotypes that arise from mutations in ATM, but little is known about the mechanisms by which the ATM protein functions. Recent studies have revealed that, like DNA-PKcs, ATM is expressed ubiquitously and is

localised predominantly in the cell nucleus (Chen and Lee, 1996; Lakin et al., 1996; Brown et al., 1997; Watters et al., 1997).

5 The realisation that ATM is a member of the PI 3-kinase family has suggested to some that the primary function of ATM might phosphorylate inositol phospholipids. Savitsky et al (1995 Science 268, 1749-1753), for example, do not  
10 of evidence suggest that ATM might have functioned in a very different way from that which we have established herein. For example, defective protein tyrosine phosphorylation and calcium mobilization in response to the triggering of B-cells and T-cells of A-T patients  
15 support the idea of defects in intra-cytoplasmic signalling pathways in A-T cells (cited in the Savitski Science paper 1995). These data are provided in the paper Khanna et al (1997; J. Biol. Chem.). This paper also summarises a variety of other data suggesting  
20 different ways in which ATM might function.

Savitsky et al (Science 1995) state that the insulin-dependent diabetes observed in some A-T patients could reflect ATM acting in an analogous way to PI  
25 3-kinase affecting glucose transport by insulin. They also discuss PI 3-kinase in terms of controlling apoptosis as a paradigm for ATM, ie. one can explain many of the features of A-T by suggesting that it works analogously to PI 3-kinase.

30 Some A-T cells have been shown to be complemented by a gene called ATDC, whose product interacts with an intermediate filament protein called vimentin, which is cytoplasmic (Brzoska et al; PNAS). They state that A-T  
35 cell lines have aberrantly aggregated actin filaments, suggesting the role of ATM lies in the cytoplasm.

We have purified ATM. We report that, ATM binds to DNA and possesses an associated protein kinase activity that is stimulated by DNA. Furthermore, we show that ATM serves as a kinase for p53 and that the sites of phosphorylation reside in functionally important regions of the p53 polypeptide. These sites are Ser15 and Thr18. We also show that DNA-PK is also capable of phosphorylating the Ser15 and Thr18 sites of p53, and that ATR phosphorylates Ser15. Further, we show that phosphorylation of these sites of p53 disrupts the interaction of p53 with Mdm-2, a protein which targets p53 for degradation within the cell.

By targeting these sites, ATM may activate p53 for DNA binding and/or cause disassociation of Mdm-2, thus stabilising p53 (leading to increased amounts of the protein) and would allow it to activate transcription.

Thr18 of p53 has to our knowledge never been shown to be phosphorylated *in vivo* or *in vitro*. This site does not conform to a characterized DNA-PK consensus phosphorylation site. Thus, our finding of phosphorylation here is totally unexpected.

Ser15 is phosphorylated by DNA-PK, but nonetheless its phosphorylation by ATM is also surprising, particularly since there are no data indicating its phosphorylation in response to DNA damage being altered in A-T cells.

Based on this and other work described below, the present invention in various aspects provides for modulation of interaction between ATM (and ATR) and p53, particularly phosphorylation of p53 by ATM and ATR, and DNA binding by these proteins, which is further shown to have a potentiating effect on phosphorylation of p53.

Various aspects of the present invention provide for the

use of ATM (or related kinases such as ATR or DNA-PK) and p53, with or without DNA, in screening methods and assays for agents which modulate interaction between ATM and p53, particularly phosphorylation of p53 by ATM.

5 Further aspects provide for modulation of interaction between ATM, or related kinases such as ATR or DNA-PK, and other molecules including a phosphorylation site homologous to those in p53 which are phosphorylated by  
10 ATM, and use of these molecules in screening methods and assays for useful agents. For simplicity, much of the present disclosure refers to ATM and p53. However, unless the context requires otherwise, every such reference should be taken to be equally applicable to the  
15 interaction between ATM and other molecules including a site homologous to one of those in p53 phosphorylated by ATM. Similarly, based on the disclosure herein, the invention extends to the use of other protein kinases which have an associated protein kinase activity capable  
20 of phosphorylating sites of p53, in particular Ser15 and Thr18. Typically, the protein kinase domain of these other kinases will share at least 30% amino acid sequence identity with the corresponding domain of ATM, more preferably at least 35% sequence identity, more  
25 preferably at least 40% sequence identity, more preferably at least 50% sequence identity, more preferably at least 70% sequence identity, still more preferably at least 90% sequence identity. Examples of such kinases are ATR (also known as FRP1, see Cimprich et  
30 al, 1996) and DNA-PKcs.

Such molecules may be identified by various means. For instance, information may be obtained about residues which are important for p53 phosphorylation by ATM using  
35 alanine scanning and deletion analysis of p53 and/or peptide fragments, for instance the N-terminal 42 amino acids or so of p53, or a fragment of around 10 amino

acids including the relevant site of phosphorylation. Mutation may be used to identify residues which affect phosphorylation and those which do not. When key residues are identified, computer sequence databases may be scanned for proteins including the same or similar pattern of residues, taking into account conservative variation in sequence (see below) as appropriate. Candidate molecules may then be used in one or more assays for phosphorylation by ATM (such as discussed below).

Identification of key residues for phosphorylation at any of the sites in p53 phosphorylated by ATM may also be used in the design of peptide and non-peptidyl agents which modulate, particularly inhibit, phosphorylation of p53 by ATM, as discussed further below.

Methods of obtaining agents able to modulate interaction between ATM and p53 (or, it must be remembered, ATR, or a related protein having a similar associated kinase activity, and other molecules including a phosphorylation site homologous to one of those phosphorylated in p53 by ATM) include methods wherein a suitable end-point is used to assess interaction in the presence and absence of a test substance. Assay systems may be used to determine ATM kinase activity, ATM DNA binding and/or ATM interaction with one or more other molecules. For phosphorylation assays, full-length p53, truncated portions of p53, or portions of p53 fused to other proteins (eg. GST), or a suitable variant or derivative of any of these may be used. Peptide phosphorylation assays may be developed using peptides that correspond to the phosphorylated regions of p53. The phosphorylation of any of the above may be assayed by any of a variety of procedures such as discussed below and may be adapted to high throughput screening approaches. Interference of DNA binding may be assayed but the inhibition of kinase

activity may be more sensitive and identify a greater breadth of inhibitors to DNA binding inhibition, and so may be preferred by the skilled operator of the present invention.

5

ATM kinase activity may be assayed for either of the two N-terminal p53 sites. When assaying for phosphorylation, DNA is preferably included in the assay system. Related but different screens may be set up for inhibitors and  
10 activators of the two sites of ATM-mediated phosphorylation event.

Generally of most interest is modulation of the phosphorylation of p53 (or other molecule) by ATM.  
15 Detailed disclosure in this respect is included below. It is worth noting, however, that combinatorial library technology provides an efficient way of testing a potentially vast number of different substances for ability to modulate an interaction with and/or activity  
20 of a polypeptide. Such libraries and their use are known in the art, for all manner of natural products, small molecules and peptides, among others. The use of peptide libraries may be preferred in certain circumstances.

25 At the cellular level, A-T cells display chromosomal instability, radiosensitivity, are impaired in p53 induction following treatment with ionising radiation, and show altered regulation of transcription factor NFkB. Thus, the wild-type ATM gene functions as a tumour  
30 suppressor, and is a suppressor of neurological degeneration and other degenerative states commonly associated with ageing.

Given the results reported herein on which the present  
35 invention is based, activators and inhibitors of ATM-associated kinase activity may be identified and appropriate agents may be obtained, designed and used for



any of a variety of purposes:

5     *A-T Therapy.* Activators of ATM or ATR function may prove to have utility in treating humans with A-T (discussed further below).

10     *Modulation of immune system function.* A-T patients display immunodeficiencies, demonstrating that ATM is required for generation of a fully functional immune system. Modulators of ATM or ATR may, therefore, be used in regulating immune system function.

15     *AIDS therapy.* It has been shown that the lymphocytes of humans entering the final stages of AIDS have shortened telomeres and this may contribute to them being no longer able to replenish the immune system. Cells of A-T patients lose their telomeres more quickly than those of normal individuals, revealing that ATM plays a positive role in telomere length homeostasis. Activators of ATM  
20     function may, therefore, find utility in treatment of individuals with AIDS through lengthening the telomeres of senescent lymphocytes in these individuals, thus allowing replenishment of the immune system.

25     *p53 therapy.* The identification of the site of p53 phosphorylated by ATM indicates that this of extreme regulatory importance. Indeed, the N-terminal sites on p53 phosphorylated by ATM reside within the region known as "conserved region I" that has been shown to function  
30     together with flanking sequences in the interaction with the protein Mdm-2 (see Kussie et al 1996; Picksley et al., 1994; Momand et al., 1992; Chen et al., 1993 and references therein). Mdm-2 serves as a negative  
35     regulator of p53 by two mechanisms. First, it masks the p53 transcriptional activation domain, stopping p53 activating genes (Momand et al., 1992). Second, Mdm-2 has been shown recently to target p53 for degradation

within the cell (Kubbutat et al., 1997; Haupt et al., 1997). Our data therefore provide an indication that phosphorylation of p53 by ATM will disrupt its interactions with Mdm-2, thus resulting in increased levels of transcriptionally active p53. This knowledge may, therefore, be utilised to generate novel therapeutic agents that target p53 - such as small molecules that, through binding to mutant p53, mimic ATM-mediated activation of this molecule.

Phosphorylation at any one or more of these sites may affect interaction of p53 with a number of proteins. Mdm2 is one particularly example given the location of Thr18 within the site on p53 to which Mdm2 binds (see e.g. Chen et al., (1993), Kussie et al., (1996), Picksley et al., (1994) and Momand et al., (1992) for characterisation of this interaction) and Ser15 which lies immediately adjacent to the minimal Mdm2 binding sequence. Indeed, a report by Shieh et al published in October 1997 indicates that phosphorylation at Ser15 can disrupt the p53-Mdm2 interaction. Phosphorylation of p53 may be used to affect interaction of p53 with any of a number of other proteins, including CBP (Gu et al.; Lill et al.), adenovirus E1B protein, which binds within the amino terminal 123 amino acids of p53 (Kao et al., 1990), with residues Leu-22 and Trp-23 playing an important role (Lin et al., 1994), transcription factors XPD (Rad3) and XPB, as well as CSB involved in strand-specific DNA repair (Wang et al., 1995), TFIIH (Xiao et al., 1994), E2F1 and DP1 (O'Connor et al., 1995), Cellular Replication Protein A (Li and Botchan, 1993), replication factor RPA (Dutta et al., 1993), WT1 (Maheswaran et al., 1993), TATA-binding protein (Seto et al., 1992, Truant et al., 1992, Martin et al., 1993), and TAF(II)40 and TAF(II)60 (Thut et al., 1995).

An assay according to the present invention as discussed

further below may determine the role of phosphorylation of p53 by ATM on any of these interactions and an agent found to be able to modulate such phosphorylation may be used to disrupt or promote any of these interactions, e.g. in a therapeutic context.

*Modulating telomere length.* A-T cells show accelerated rates of telomere shortening (Metcalfe et al., 1996, *Nature Genetics* 13, 350-353). Thus, regulators of ATM activity may be used to control telomere length. ATM does not appear to be part of the telomerase enzyme itself (Metcalfe et al. shows that telomerase levels are normal in A-T cells; also, our data and the data of Pandita et al. 1995 show that A-T cells have somewhat shortened telomeres but do not have repressed levels of telomerase). Thus, ATM works not as part of telomerase but as part of a telomere length homeostatic mechanism. It is therefore likely that anti-ATM drugs will work synergistically with anti-telomerase drugs.

*Ageing.* A-T patients display enhanced rates of ageing, display a number of symptoms associated with increased age (neurological deterioration, cancers, immunological deficiencies etc), and their cells show shortened lifespan in culture. Agents that modulate ATM activity may therefore be used to treat/prevent disease states associated with premature and normal ageing.

*Tumour/Cancer therapy.* This is discussed below. Drugs that modulate ATM action may be used to treat A-T patients; treat cancer - through affecting cellular growth capacity by shortening cells telomeres; manipulate the immune system - A-T patients are somewhat immunodeficient; treat cancer - radiosensitization of tumours etc (see below). Also, ATM modulators may be used to limit cell growth potential by affecting telomere length etc. The linkage to p53 may allow p53 therapy,

activating p53 in cancer cells, which may lead to cell growth arrest and/or cell death via apoptosis or another route.

- 5 Activators of ATM (or ATR, DNA-PK or related kinases) may be used, for example, to inhibit cell proliferation by activating cell cycle checkpoint arrest in the absence of cellular damage, which may be used in the treatment of tumours, cancer, psoriasis, arteriosclerosis and other
- 10 hyper-proliferative disorders. Activators may be employed to activate p53 in cells without damaging the cells. Cells of a patient may be treated so that normal cells (p53+) stop growing and are thus refractory to killing by administration of a drug that kills cells via
- 15 interfering with cell division or DNA replication, while tumour cells (many of which are p53 negative) do not arrest and are consequently selectively killed by the aforementioned agents. By way of example, ATM activators include peptides capable of recognising and binding to
- 20 both ATM and p53 but which do not interfere with the phosphorylation of the Ser15 and Thr18 sites of p53, or substances capable of activating ATM in a similar manner to the activation observed using DNA.
- 25 Cancer radiotherapy and chemotherapy may be augmented using agents in accordance with the present invention. Ionising radiation (IR) and radiomimetic drugs are used commonly to treat cancers, and kill cancer cells predominantly via inflicting DNA damage. Cells deficient
- 30 in ATM are hypersensitive to ionising radiation and radiomimetics. Thus, inhibitors of the ATM will hypersensitise cells to the killing effects of ionising radiation and radiomimetics. ATM inhibitors may thus be used as adjuncts in cancer radiotherapy and chemotherapy.
- 35 Cell growth capacity may be modulated e.g. in treatment of cancer, ageing, and AIDS. It is established that ATM

plays a crucial role in controlling the length of telomeric chromosomal ends (Metcalf et al.). Telomeric ends in most normal cell types shorten at each cell division, and cells with excessively shortened telomeres are unable to divide. Thus, telomeres are thought to function as a "division counting apparatus" that limits the proliferative capacity of most normal mammalian cells. Inhibitors of ATM function may, therefore, have utility in preventing cancer progression by limiting the growth potential of cancerous or pre-cancerous cells. Activators of ATM may be used to release senescent cells from growth arrest and may thus have utility in treatments of aged individuals. In addition, it has been shown recently that the lymphocytes of humans entering the final stages of AIDS have shortened telomeres and this may contribute to these cells being no longer able to proliferate and replenish the immune system. ATM activators may, therefore, result in lengthening of the telomeres of such cells and restoring their proliferative capacity.

Interaction between ATM and p53 may be inhibited by inhibition of the production of the relevant protein. For instance, production of one or more of these components may be inhibited by using appropriate nucleic acid to influence expression by antisense regulation. The use of anti-sense genes or partial gene sequences to down-regulate gene expression is now well-established. Double-stranded DNA is placed under the control of a promoter in a "reverse orientation" such that transcription of the "anti-sense" strand of the DNA yields RNA which is complementary to normal mRNA transcribed from the "sense" strand of the target gene. The complementary anti-sense RNA sequence is thought then to bind with mRNA to form a duplex, inhibiting translation of the endogenous mRNA from the target gene into protein. Whether or not this is the actual mode of

action is still uncertain. However, it is established fact that the technique works.

Another possibility is that nucleic acid is used which on transcription produces a ribozyme, able to cut nucleic acid at a specific site - thus also useful in influencing gene expression. Background references for ribozymes include Kashani-Sabet and Scanlon, 1995, *Cancer Gene Therapy*, 2(3): 213-223, and Mercola and Cohen, 1995, *Cancer Gene Therapy*, 2(1), 47-59.

Thus, various methods and uses of modulators, which inhibit or potentiate interaction of ATM and p53, particularly phosphorylation of p53 by ATM, are provided as further aspects of the present invention. The purpose of disruption, interference with or modulation of interaction between ATM and p53, particularly the phosphorylation of p53 by ATM may be to modulate any activity mediated by virtue of such interaction, as discussed above and further below.

Various aspects of the present invention relate to modulation of interaction between ATM and DNA. Such interaction is established here we believe for the first time, and is further shown to have an effect on p53 phosphorylation by ATM. It was surprising that ATM is a DNA binding protein, as there are data suggesting that it is associated with microsomal membranes in the cytoplasm (Watters et al, 1997 and Brown et al, 1997; show ATM is also present in cytoplasmic vesicles) and A-T cells have also been reported to be defective in signalling from the cell membrane in B- and T-cells (see above). It was furthermore surprising that ATM would bind DNA so well. The purification method used and described below does not purify a variety of other (known) DNA binding factors, yet ATM is purified very selectively (about 100-fold in a single step) using a DNA affinity chromatography

procedure.

5 The present invention provides in one aspect the use of DNA for purifying ATM or ATR. In further aspects, the present invention provides for the use of DNA in assays for activity of ATM or ATR, particularly phosphorylation of p53 (or other molecule).

10 We have also purified ATM and ATR via another surprising route, using nitrilo-tri-acetic acid (NTA) agarose. NTA has 4 chelating sites for  $\text{Ni}^{2+}$ . Another  $\text{Ni}^{2+}$  matrix, iminodiacetic acid (IDA) agarose (with 3-chelating sites for  $\text{Ni}^{2+}$ ) we have found to bind ATM only weakly. These  $\text{Ni}^{2+}$  matrices are generally used interchangeably to purify  
15 proteins that chelate metal ions, usually, via a run of His residues (usually 6 give best binding). ATM does not have a run of 6, 5 or even 4 His residues, so it is surprising that ATM or ATR is purifiable by the Ni-linked columns. Furthermore, since the two matrices are  
20 generally used interchangeably, it is further surprising that ATM binds to the NTA well but only poorly to the IDA matrix.

25 ATM no doubt works in concert with other factors in the detection and signalling of DNA damage. Indeed, although our data reveal that ATM possesses intrinsic DNA-stimulated p53 kinase function, we have observed repeatedly that the presence of additional polypeptides correlates with increased ATM activity. Thus, our most  
30 highly purified preparations have considerably less activity than preparations containing an equivalent amount of ATM but also possessing additional co-purifying polypeptides. It is likely that these serve to help tether ATM to the DNA and/or trigger its kinase activity  
35 by altering the conformation of the ATM polypeptide. Accordingly, references to ATM, or a protein having a associated kinase activity, include both purified ATM (or

the related protein) and ATM (or the related protein) in combination with associated polypeptides or co-factors present in preparations as obtainable by the methods described herein.

5 Assays according to the present invention may be used in the identification of such additional polypeptides, for example by assaying for protein fractions that stimulate ATM activity. The use of ATM or ATR in identifying  
10 and/or obtaining cofactors which (e.g. naturally) enhance its kinase activity is further provided by the present invention. ATM activity may under certain circumstances be masked by one or more factors (see discussion section below). Accordingly, the present invention also provides  
15 for the use of ATM in identifying and/or obtaining such factors.

Protein or other co-factors of ATM, e.g. which enhance ATM kinase activity, may be used in the design of  
20 inhibitors of this, providing another route for modulating ATM activity. This may similarly be used to provide a route to deriving agents that activate ATM, e.g. by inhibiting one or more repressors of ATM activity.

#### 25 *Brief Description of the Figures*

Figure 1: ATM binds to DNA. (A) ATM binds to a dsDNA oligonucleotide. HeLa nuclear extract was bound to either streptavidin iron oxide beads (-DNA) or  
30 streptavidin iron oxide beads bearing a 50-mer ds DNA oligonucleotide (+DNA). After extensive washing, ATM was eluted from DNA in 500 mM KCl. Eluted proteins were subjected to 7% SDS-PAGE and ATM visualised by Western blotting using ATM.B antiserum. (B) Binding of ATM is  
35 dependent on DNA length. ATM enriched extract was bound to streptavidin iron oxide beads attached to ds DNA of various sizes (15, 25, 50 or 75 bp). After extensive



washing, ATM was eluted by sequential washes with 100, 250 and 500 mM KCl. Eluates were analysed as in (A).

(C) ATM binds DNA containing a variety of different architectures. ATM enriched extract was bound to streptavidin iron oxide beads bound to either ss or ds DNA containing a nick, ds/ss transition, gap or 10 bp insertion. Washing, elution and ATM detection was as in (B).

Figure 2: Purification of ATM from HeLa cell nuclear extract. (A) ATM Purification strategy. HeLa nuclear extract was subjected to ion exchange chromatography using Q-Sepharose and peak ATM fractions, eluting between 160-200 mM KCl, were passed over heparin-agarose ion exchange resin. ATM fractions eluting from heparin-agarose between 200-220 mM KCl were pooled and subjected to DNA affinity purification and elution from DNA-bearing beads at 500 mM KCl resulting in an essentially homogeneous preparation of ATM. (B) Purification of ATM to essential homogeneity. Equivalent volumes (5  $\mu$ l) of HeLa cell nuclear extract (50  $\mu$ g protein), or pooled fractions following Q-sepharose, Heparin-agarose or DNA affinity chromatography were subjected to 7% SDS-PAGE and proteins visualised by silver staining (upper panel). Fractions were also subjected to Western blot analysis (lower panel) using antibodies raised against ATM, DNA-PK<sub>cs</sub>, Ku70 plus Ku80 or the 70kDa subunit of RPA, as indicated.

Figure 3: Purified ATM possesses an associated p53 kinase activity. (A) Analysis of putative ATM substrates. DNA-PK<sub>cs</sub> (60 ng), Ku (100 ng), Sp1 (100 ng), p53 (100 ng), RPA-p34 (100 ng) or PCNA (100 ng) were used in kinase reactions in conjunction with approximately 11 fmole of purified ATM (see Experimental Procedures). Proteins were resolved on either 7% (left panel) or 10% (right panel) polyacrylamide gels and phosphorylated

proteins detected by autoradiography. (B) Analysis of total proteins immuno-precipitated from purified ATM preparations. Purified ATM was biotinylated and subjected to immunoprecipitation using either pre-immune sera, or ATM antisera raised against amino acid residues 1980-2337 (ATM.B) or the N-terminus (ATM.N) of ATM. Precipitated proteins were resolved on 7.8% polyacrylamide gels and, after transfer to nitrocellulose, total precipitated proteins were detected by probing filters with streptavidin-conjugated horseradish peroxidase. (C) Immunoprecipitated ATM possesses p53 kinase activity. Purified ATM was immunoprecipitated using pre-immune sera, or anti-ATM antisera ATM.B or ATM.N. Following immunoprecipitation, kinase reactions were performed either in the presence or absence of p53 as indicated. Phosphorylated proteins were resolved on 10% polyacrylamide gels and detected by autoradiography.

Figure 4: A DNA-stimulated protein kinase activity co-purifies with ATM. (A) ATM associated kinase activity is stimulated by linear DNA containing multiple p53 binding sites. Purified ATM, DNA-PK or cyclin A/cdk2 (11 fmole), as indicated, were used in kinase reactions containing p53 either in the absence (-) or presence of 0.03, 0.3 or 3 fmole of linear DNA bearing multiple p53 binding sites (pG<sub>13</sub>CAT). Proteins were resolved on 10% polyacrylamide gels and phosphorylated proteins visualised by autoradiography. (B) ATM associated kinase activity does not require DNA ends. *In vitro* kinase reactions containing 11 fmole of purified ATM in conjunction with p53 were performed in either the absence (-) or presence of 0.03, 0.3 or 30 fmole of linear or supercoiled pG<sub>13</sub>CAT DNA. Proteins were detected as in (A).

Figure 5: ATM phosphorylates p53 at Ser15 and Thr18 in the presence of DNA. Kinase reactions employing ATM and

p53 were performed in the presence and absence of DNA. These studies revealed phosphorylation of p53 was increased in the presence of DNA. (A,B) Bands corresponding to  $^{32}\text{P}$ -labelled p53 were excised from a gel, digested with trypsin, and chromatographed on a Vydac 218TP54 C18 column (see Experimental Procedures). Purified p53 fractions phosphorylated by ATM preparations in the presence, but not in the absence, of DNA (peptides 2a, 2b and 2c) were subjected to peptide sequence analysis as described in Experimental Procedures; radioactivity was measured after each cycle of Edman degradation. The putative amino acid sequence of the p53 peptide showing incorporation of  $^{32}\text{P}$  is indicated in panel C. (D) Tryptic peptide map of p53 phosphorylated by DNA-PK in the presence of DNA. Kinase reactions containing DNA-PK and p53 were performed in the presence of linear DNA and  $^{32}\text{P}$ -labelled p53 was analysed as in (A,B), again revealing phosphorylation at Ser15 and Thr18.

Figure 6a shows the amino acid sequence of human ATM, with the kinase domain marked by underlining. Figure 6b shows the ATM nucleic acid sequence with the initiation codon underlined.

Figure 7a shows the amino acid sequence of human p53 with residues phosphorylated by ATM marked by underlining. Figure 7b shows the p53 nucleic acid sequence with the initiation codon underlined.

Figure 8a shows the amino acid sequence of human ATR (FRP-1). Figure 8b shows the ATR nucleic acid sequence with the initiation codon underlined.

Figure 9a shows the amino acid sequence of DNA-PKcs. Figure 9b shows the DNA-PK nucleic acid sequence with the initiation codon underlined.

Figure 10 shows fractionation of two DNA activated kinase activities in HeLa nuclear cell extract capable of phosphorylating Ser15 of p53. Top panel; a Western immuno-blot was performed with antibodies that specifically recognise p53 phosphorylated on Ser15 on reactions in which fractions generated when HeLa cell nuclear extract was fractionated on Q-sepharose were incubated with p53 and ATP in the presence of sonicated calf thymus DNA. Middle panel; the same set of fractions were tested for DNA-PKcs by using an anti-DNA-PKcs antiserum in western immuno-blot analysis. Lower panel: the same set of fractions were tested for the presence of ATR by using an anti-ATR antiserum in western immuno-blot analysis. Additional studies revealed that both activities detected are stimulated by DNA.

Figure 11 shows DNA activated kinase activity (activity 1) co-fractionates with ATR. Activity peak 1 was fractionated further on DNA-cellulose followed by chromatography on Heparin-agarose. Bottom panel; the final set of fractions was tested for p53 kinase activity via incubation with p53, ATP and DNA and then analysis by SDS-polyacrylamide gel electrophoresis and Western immuno-blotting using the p53 Ser15-specific antibodies. Top panel; a silver-stain of an SDS-polyacrylamide gel of the same set of fractions tested for p53 kinase activity. ATR is indicated with an arrow.

The present invention in various aspects provides for modulating, interfering with or interrupting, increasing or potentiating interaction between the ATM protein and p53, particularly phosphorylation of p53 by ATM, using an appropriate agent. As noted, it having now been established for the first time that ATM is a protein kinase, it is highly likely to act on other molecules, particularly proteins including a site which is homologous to one of the sites in p53 phosphorylated by

ATM. The present invention further extends to the use of proteins having an associated kinase activity similar to ATM, especially DNA-PK and ATR. The present invention extends to modulation of such phosphorylation and this should be borne in mind when considering the disclosure herein which for convenience uses p53 for illustrative purposes, and as a preferred embodiment in certain contexts.

An agent capable of modulating interaction between ATM and p53 may be capable of blocking interaction between a site located within amino acid residues including Ser15 or Thr18.

In addition to interacting at the site of phosphorylation of p53, ATM and p53 may interact at one or more other sites within either or both proteins. Affecting interaction at such a site may have an effect on phosphorylation of p53 by ATM. Various fragments and derivatives of the proteins, particular of p53, may be used to analyse this, using techniques such as alanine scanning and deletion analysis. The present invention encompasses modulation of interaction between ATM and p53 at any site, preferably resulting in modulation of p53 phosphorylation by ATM.

The full amino acid sequence of the ATM protein has been elucidated and is set out in Savitsky et al 1995a, 1995b, and Figure 6a, of which the amino acid residue numbering is used. The kinase domain is marked in Figure 6a. The p53 amino acid sequence is shown in Figure 7a, of which the amino acid residue numbering is used. These sequences are human sequences. ATM and p53 are conserved among vertebrates, particular mammals - see e.g. Figure 2 of Soussi et al. For p53 conservation in the regions of the residues shown herein to be phosphorylated by ATM - so the present invention extends to use in any of its

aspects of other vertebrate, particularly mammalian, p53 and/or ATM, e.g. primate, such as monkey, rodent, such as mouse or rat, pig, horse, cow, sheep, goat, dog, cat, and so on. The amino acid and nucleic acid sequences of ATR (also known as FRP1) are set out in Cimpich et al, 1996. The amino acid sequence is reproduced as Figure 8a. The amino acid sequence of DNA-PK is provided in Hartley et al, 1995 and is set out in Figure 9a. The nucleic acid sequences of these proteins are also included as Figures 6b, 7b, 8b and 9b.

Agents useful in accordance with the present invention may be identified by screening techniques which involve determining whether an agent under test inhibits or disrupts the interaction of ATM protein or a suitable fragment thereof (e.g. including amino acid residues of the kinase domain, as marked on Figure 6, or a smaller fragment of any of these regions) of ATM, with p53 or a fragment thereof, or a suitable analogue, fragment or variant thereof. One class of preferred fragments of p53 are those which include one or both of the phosphorylation sites at Ser15 or Thr18.

Suitable fragments of ATM or p53 include those which include residues which interact with the counterpart protein. Smaller fragments, and analogues and variants of this fragment may similarly be employed, e.g. as identified using techniques such as deletion analysis or alanine scanning.

Thus, the present invention provides a peptide fragment of ATM which is able to interact with p53 and/or inhibit interaction between ATM and p53, particularly phosphorylation of p53 by ATM, and provides a peptide fragment of p53 which is able to interact with ATM and/or inhibit interaction between p53 and ATM, particularly phosphorylation of p53 by ATM, such peptide fragments

being obtainable by means of deletion analysis and/or alanine scanning of the relevant protein - making an appropriate mutation in sequence, bringing together a mutated fragment of one of the proteins with the other or a fragment thereof and determining interaction, preferably phosphorylation of p53 or fragment thereof. In preferred embodiments, the peptide is short, as discussed below, and may be a minimal portion that is able to interact with the relevant counterpart protein and/or inhibit the relevant interaction.

Screening methods and assays are discussed in more detail below.

One class of agents that can be used to disrupt the interaction of ATM and p53 are peptides based on the sequence motifs of ATM or p53 that interact with counterpart p53 or ATM (as discussed already above). Such peptides tend to be short, and may be about 40 amino acids in length or less, preferably about 35 amino acids in length or less, more preferably about 30 amino acids in length, or less, more preferably about 25 amino acids or less, more preferably about 20 amino acids or less, more preferably about 15 amino acids or less, more preferably about 10 amino acids or less, or 9, 8, 7, 6, 5 or less in length. The present invention also encompasses peptides which are sequence variants or derivatives of a wild type ATM or p53 sequence, but which retain ability to interact with p53 or ATM (respectively, as the case may be) and/or ability to modulate interaction between ATM and p53, particularly phosphorylation of p53 by ATM.

Instead of using a wild-type ATM or p53 fragment, a peptide or polypeptide may include an amino acid sequence which differs by one or more amino acid residues from the wild-type amino acid sequence, by one or more of

addition, insertion, deletion and substitution of one or more amino acids. Thus, variants, derivatives, alleles, mutants and homologues, e.g. from other organisms, are included.

5

Preferably, the amino acid sequence shares homology with a fragment of the relevant ATM or p53 fragment sequence shown preferably at least about 30%, or 40%, or 50%, or 60%, or 70%, or 75%, or 80%, or 85%, 90% or 95% homology. Thus, a peptide fragment of ATM or p53 may include 1, 2, 3, 4, 5, greater than 5, or greater than 10 amino acid alterations such as substitutions with respect to the wild-type sequence. Preferably, the peptide fragments of ATM are based on the sequence of all or part of the kinase domain as shown in figure 6. Preferably, the p53 fragments are based on the N-terminal sequence of the molecule around the sites phosphorylated by ATM, i.e. comprising the amino acid motif PPLSQETFS, or more generally, the motif SxxT, where x is any amino acid.

15  
20

A derivative of a peptide for which the specific sequence is disclosed herein may be in certain embodiments the same length or shorter than the specific peptide. In other embodiments the peptide sequence or a variant thereof may be included in a larger peptide, as discussed above, which may or may not include an additional portion of ATM or p53. 1, 2, 3, 4 or 5 or more additional amino acids, adjacent to the relevant specific peptide fragment in ATM or p53, or heterologous thereto may be included at one end or both ends of the peptide.

25  
30

(It should not be forgotten that references to ATM and p53 apply equally to ATM and related proteins such as ATR and DNA-PK and other proteins including a phosphorylation site homologous to one in p53 phosphorylated by ATM.)

35

As is well-understood, homology at the amino acid level



is generally in terms of amino acid similarity or identity. Similarity allows for "conservative variation", i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. Similarity may be as defined and determined by the TBLASTN program, of Altschul et al. (1990) *J. Mol. Biol.* 215: 403-10, which is in standard use in the art. Homology may be over the full-length of the relevant peptide or over a contiguous sequence of about 5, 10, 15, 20, 25, 30, 35, 50, 75, 100 or more amino acids, compared with the relevant wild-type amino acid sequence.

As noted, variant peptide sequences and peptide and non-peptide analogues and mimetics may be employed, as discussed further below.

Various aspects of the present invention provide a substance, which may be a single molecule or a composition including two or more components, which includes a peptide fragment of ATM or p53 which includes a sequence as recited in Figure 6 or Figure 7, particularly within the ATM kinase domain marked in Figure 6, a peptide consisting essentially of such a sequence, a peptide including a variant, derivative or analogue sequence, or a non-peptide analogue or mimetic which has the ability to interact with ATM or p53 and/or modulate, disrupt or interfere with interaction between ATM or p53.

Variants include peptides in which individual amino acids can be substituted by other amino acids which are closely related as is understood in the art and indicated above.

Non-peptide mimetics of peptides are discussed further

below.

As noted, a peptide according to the present invention and for use in various aspects of the present invention may include or consist essentially of a fragment of ATM or p53 as disclosed, such as a fragment whose sequence is shown in Figure 6 or Figure 7, respectively. Where one or more additional amino acids are included, such amino acids may be from ATM or p53 or may be heterologous or foreign to ATM or p53. A peptide may also be included within a larger fusion protein, particularly where the peptide is fused to a non-ATM or p53 (i.e. heterologous or foreign) sequence, such as a polypeptide or protein domain.

The invention also includes derivatives of the peptides, including the peptide linked to a coupling partner, e.g. an effector molecule, a label, a drug, a toxin and/or a carrier or transport molecule, and/or a targeting molecule such as an antibody or binding fragment thereof or other ligand. Techniques for coupling the peptides of the invention to both peptidyl and non-peptidyl coupling partners are well known in the art. In one embodiment, the carrier molecule is a 16 aa peptide sequence derived from the homeodomain of *Antennapedia* (e.g. as sold under the name "Penetratin"), which can be coupled to a peptide via a terminal Cys residue. The "Penetratin" molecule and its properties are described in WO 91/18981.

Peptides may be generated wholly or partly by chemical synthesis. The compounds of the present invention can be readily prepared according to well-established, standard liquid or, preferably, solid-phase peptide synthesis methods, general descriptions of which are broadly available (see, for example, in J.M. Stewart and J.D. Young, *Solid Phase Peptide Synthesis*, 2nd edition, Pierce Chemical Company, Rockford, Illinois (1984), in M.

Bodanzsky and A. Bodanzsky, The Practice of Peptide Synthesis, Springer Verlag, New York (1984); and Applied Biosystems 430A Users Manual, ABI Inc., Foster City, California), or they may be prepared in solution, by the liquid phase method or by any combination of solid-phase, liquid phase and solution chemistry, e.g. by first completing the respective peptide portion and then, if desired and appropriate, after removal of any protecting groups being present, by introduction of the residue X by reaction of the respective carbonic or sulfonic acid or a reactive derivative thereof.

Another convenient way of producing a peptidyl molecule according to the present invention (peptide or polypeptide) is to express nucleic acid encoding it, by use of nucleic acid in an expression system.

Accordingly the present invention also provides in various aspects nucleic acid encoding the polypeptides and peptides of the invention.

Generally, nucleic acid according to the present invention is provided as an isolate, in isolated and/or purified form, or free or substantially free of material with which it is naturally associated, such as free or substantially free of nucleic acid flanking the gene in the human genome, except possibly one or more regulatory sequence(s) for expression. Nucleic acid may be wholly or partially synthetic and may include genomic DNA, cDNA or RNA. Where nucleic acid according to the invention includes RNA, reference to the sequence shown should be construed as reference to the RNA equivalent, with U substituted for T.

Nucleic acid sequences encoding a polypeptide or peptide in accordance with the present invention can be readily prepared by the skilled person using the information and

references contained herein and techniques known in the art (for example, see Sambrook, Fritsch and Maniatis, "Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, and Ausubel et al, Short  
5 Protocols in Molecular Biology, John Wiley and Sons, 1992), given the nucleic acid sequence and clones available. These techniques include (i) the use of the polymerase chain reaction (PCR) to amplify samples of such nucleic acid, e.g. from genomic sources, (ii)  
10 chemical synthesis, or (iii) preparing cDNA sequences. DNA encoding ATM or p53 fragments may be generated and used in any suitable way known to those of skill in the art, including by taking encoding DNA, identifying suitable restriction enzyme recognition sites either side  
15 of the portion to be expressed, and cutting out said portion from the DNA. The portion may then be operably linked to a suitable promoter in a standard commercially available expression system. Another recombinant approach is to amplify the relevant portion of the DNA  
20 with suitable PCR primers. Modifications to the ATM or p53 sequences can be made, e.g. using site directed mutagenesis, to lead to the expression of modified ATM or p53 peptide or to take account of codon preference in the host cells used to express the nucleic acid.

25 In order to obtain expression of the nucleic acid sequences, the sequences can be incorporated in a vector having one or more control sequences operably linked to the nucleic acid to control its expression. The vectors  
30 may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the polypeptide or peptide is produced as a fusion and/or nucleic acid encoding secretion signals so that the polypeptide  
35 produced in the host cell is secreted from the cell. Polypeptide can then be obtained by transforming the vectors into host cells in which the vector is

functional, culturing the host cells so that the polypeptide is produced and recovering the polypeptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of E. coli, yeast, and eukaryotic cells such as COS or CHO cells.

Thus, the present invention also encompasses a method of making a polypeptide or peptide (as disclosed), the method including expression from nucleic acid encoding the polypeptide or peptide (generally nucleic acid according to the invention). This may conveniently be achieved by growing a host cell in culture, containing such a vector, under appropriate conditions which cause or allow expression of the polypeptide. Polypeptides and peptides may also be expressed in in vitro systems, such as reticulocyte lysate.

Systems for cloning and expression of a polypeptide in a variety of different host cells are well known. Suitable host cells include bacteria, eukaryotic cells such as mammalian and yeast, and baculovirus systems. Mammalian cell lines available in the art for expression of a heterologous polypeptide include Chinese hamster ovary cells, HeLa cells, baby hamster kidney cells, COS cells and many others. A common, preferred bacterial host is E. coli.

Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. phage, or phagemid, as appropriate. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press. Many known techniques and

5 protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons, 1992.

10 Thus, a further aspect of the present invention provides a host cell containing heterologous nucleic acid as disclosed herein.

15 The nucleic acid of the invention may be-integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques. The nucleic acid may be on an extra-chromosomal vector within the cell, or otherwise identifiably heterologous or foreign to the cell.

20 A still further aspect provides a method which includes introducing the nucleic acid into a host cell. The introduction, which may (particularly for *in vitro* introduction) be generally referred to without limitation as "transformation", may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g. 25 vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. As an alternative, direct injection of the nucleic acid could be employed.

35 Marker genes such as antibiotic resistance or sensitivity genes may be used in identifying clones containing

nucleic acid of interest, as is well known in the art.

5 The introduction may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells (which may include cells actually transformed although more likely the cells will be descendants of the transformed cells) under conditions for expression of the gene, so that the encoded polypeptide (or peptide) is produced. If the polypeptide is expressed coupled to an  
10 appropriate signal leader peptide it may be secreted from the cell into the culture medium. Following production by expression, a polypeptide or peptide may be isolated and/or purified from the host cell and/or culture medium, as the case may be, and subsequently used as desired,  
15 e.g. in the formulation of a composition which may include one or more additional components, such as a pharmaceutical composition which includes one or more pharmaceutically acceptable excipients, vehicles or carriers (e.g. see below).

20 Introduction of nucleic acid encoding a peptidyl molecule according to the present invention may take place in vivo by way of gene therapy, to disrupt or interfere with interaction between ATM or p53

25 Thus, a host cell containing nucleic acid according to the present invention, e.g. as a result of introduction of the nucleic acid into the cell or into an ancestor of the cell and/or genetic alteration of the sequence  
30 endogenous to the cell or ancestor (which introduction or alteration may take place in vivo or ex vivo), may be comprised (e.g. in the soma) within an organism which is an animal, particularly a mammal, which may be human or non-human, such as rabbit, guinea pig, rat, mouse or  
35 other rodent, cat, dog, pig, sheep, goat, cattle or horse, or which is a bird, such as a chicken.  
Genetically modified or transgenic animals or birds

comprising such a cell are also provided as further aspects of the present invention.

5 This may have a therapeutic aim. (Gene therapy is discussed below). Also, the presence of a mutant, allele, derivative or variant sequence within cells of an organism, particularly when in place of a homologous endogenous sequence, may allow the organism to be used as a model in testing and/or studying substances which  
10 modulate activity of the encoded polypeptide *in vitro* or are otherwise indicated to be of therapeutic potential. Knock-out mice, for instance, may be used to test for radiosensitivity. Conveniently, however, at least preliminary assays for such substances may be carried out  
15 *in vitro*, that is within host cells or in cell-free systems. Where an effect of a test compound is established on cells *in vitro*, those cells or cells of the same or similar type may be grafted into an appropriate host animal for *in vivo* testing.

20 For instance, p53 function or activity may be measured in an animal system such as a tumour model, e.g. involving a xenograft, relying on active p53. The animal may be subject to radio- or chemo-therapy and a test substance  
25 administered. An augmentation of the reaction in the animal to the radio- or chemo-therapy may be indicative of blocking of ATM phosphorylation of p53.

30 Suitable screening methods are conventional in the art. They include techniques such as radioimmunosassay, scintillation proximity assay and ELISA methods. Suitably either the ATM protein or fragment or p53 or fragment, or an analogue, derivative, variant or functional mimetic thereof, is immobilised whereupon the  
35 other is applied in the presence of the agents under test. In a scintillation proximity assay, a biotinylated protein fragment may be bound to



streptavidin coated scintillant - impregnated beads (produced by Amersham). Binding of radiolabelled peptide is then measured by determination of radioactivity induced scintillation as the radioactive peptide binds to the immobilized fragment. Agents which intercept this are thus inhibitors of the interaction. Further ways and means of screening for agents which modulate interaction between ATM and p53 are discussed below.

In one general aspect, the present invention provides an assay method for a substance with ability to modulate, e.g. disrupt or interfere with interaction between ATM and p53, the method including:

(a) bringing into contact a substance according to the invention including a peptide fragment of ATM, or a protein having an associated kinase activity, or a derivative, variant or analogue thereof as disclosed, a substance including the relevant fragment of p53 or a variant, derivative or analogue thereof.

A test compound which disrupts, reduces, interferes with or wholly or partially abolishes interaction between said substances (e.g. including a ATM fragment and including a p53 fragment), and which may modulate ATM and/or p53 activity, may thus be identified.

Agents which increase or potentiate interaction between the two substances may be identified using conditions which, in the absence of a positively-testing agent, prevent the substances interacting.

Another general aspect of the present invention provides an assay method for a substance able to interact with the relevant region of ATM or p53 as the case may be, the method including:

(a) bringing into contact a substance which includes a peptide fragment of ATM or a protein having an

associated kinase activity which interacts with p53 as disclosed, or which includes a peptide fragment of p53 which interacts with ATM or a protein having an associated kinase activity, or a variant, derivative or analogue of such peptide fragment, as disclosed, and a test compound; and,

(b) determining interaction between said substance and the test compound.

A test compound found to interact with the relevant portion of ATM may be tested for ability to modulate, e.g. disrupt or interfere with, ATM interaction with p53 and/or ability to affect p53 and/or ATM activity or other activity mediated by ATM or p53 as discussed already above.

Similarly, a test compound found to interact with the relevant portion of p53 may be tested for ability to modulate, e.g. disrupt or interfere with, p53 interaction with ATM and/or ability to affect ATM and/or p53 activity or other activity mediated by p53 or ATM as discussed elsewhere herein.

Another general aspect of the present invention provides an assay method for a substance able to affect p53 activity, the method including:

- (a) bringing into contact p53 and a test compound; and,
- (b) determining p53 activity.

p53 activity may be determined in the presence and absence of ATM to allow for an effect of a test compound on activity to be attributed to an effect on interaction between p53 and ATM, preferably phosphorylation of p53 by ATM (discussed further below).

p53 activities which may be determined include induction

of expression of a protein such as p21 (WAF1), cellular sensitivity to ionizing radiation, p53-induced apoptosis activity, p53-induced anti-proliferative activity, p53-induced senescence of cells

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In assaying for agents able to modulate phosphorylation of p53 by ATM, suitable fragments of p53 may be employed including any of the sites of such phosphorylation.

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Where it is desired to determine phosphorylation at the Ser15 and/or Thr18 site, DNA will generally be included in the assay system to stimulate the requisite kinase activity of ATM. As noted, the present invention extends also to non-human p53 and phosphorylation at sites equivalent to those of human p53 identified herein.

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Thus, the assays may employ derivatives of full length p53 or the p53 fragments including the phosphorylation sites at Ser15 and/or Thr18.

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The present invention further provides the use of DNA for stimulating phosphorylation of p53 by ATM, e.g. in an assay but also in many other contexts. Such phosphorylation may include at the Ser15 and/or Thr18 site of human p53 or equivalent site in p53 of another species, particularly of a vertebrate such as a mammal.

25

An assay according to the present invention may include an inhibitor of DNA-PKcs kinase activity, to avoid complications of redundant phosphorylation by that kinase. Such an inhibitor of DNA-PKcs kinase activity might not affect ATM kinase activity.

30

Further assays according to the present invention are for agents which modulate DNA binding by ATM. Inhibitors and/or activators may be screened using appropriate conditions for determination of DNA binding by ATM.

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Thus, a further aspect of the present invention provides

an assay method for a compound able to affect DNA binding by ATM or a protein having an associated kinase activity, the method including:

(a) bringing into contact a substance which is ATM or a protein having an associated kinase activity, or a fragment, variant or derivative thereof able to bind DNA, DNA and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of DNA binding by ATM or the protein having an associated kinase activity, said substance binds said DNA; and,

(b) determining binding between said substance and said DNA.

Activators of DNA binding by ATM may similarly be identified using an assay method wherein said substance, the DNA and the test compound are brought together under conditions wherein in the absence of the test compound being a potentiator of DNA binding by ATM, the substance does not bind the DNA. Activators include substances which activate ATM associated kinase activity in the absence of DNA or substances which enhance the interaction of ATM and p53, both of which may allow the induction of a p53 response in the absence of DNA damage, e.g. as caused by irradiation.

DNA binding may be determined using any suitable technique, including an electrophoretic mobility shift assay (EMSA), UV protein-DNA crosslinking, chemical or DNaseI footprinting, and so on.

Determination of DNA binding by ATM may be performed in conjunction with determination of phosphorylation, sequentially or simultaneously. For instance a preliminary screen may identify molecules which modulate DNA binding by ATM and such substances may then be used in assays to determine their ability (or not) to modulate phosphorylation of p53. The converse, in which ability

to modulate phosphorylation is determined prior to ability to modulate ATM DNA binding, is also possible, as is to run two assays in parallel.

5 Preliminary assays *in vitro* may be followed by, or run in parallel with, *in vivo* assays.

Of course, the person skilled in the art will design any appropriate control experiments with which to compare  
10 results obtained in test assays.

Performance of an assay method according to the present invention may be followed by isolation and/or manufacture and/or use of a compound, substance or molecule which  
15 tests positive for ability to modulate interaction between ATM and p53 and/or inhibit ATM or p53 activity or a mediated activity.

The precise format of an assay of the invention may be  
20 varied by those of skill in the art using routine skill and knowledge. For example, interaction between substances may be studied *in vitro* by labelling one with a detectable label and bringing it into contact with the other which has been immobilised on a solid support.  
25 Suitable detectable labels, especially for peptidyl substances include <sup>35</sup>S-methionine which may be incorporated into recombinantly produced peptides and polypeptides. Recombinantly produced peptides and polypeptides may also be expressed as a fusion protein  
30 containing an epitope which can be labelled with an antibody.

The protein which is immobilized on a solid support may be immobilized using an antibody against that protein  
35 bound to a solid support or via other technologies which are known *per se*. A preferred *in vitro* interaction may utilise a fusion protein including glutathione-S-

transferase (GST). This may be immobilized on glutathione agarose beads. In an *in vitro* assay format of the type described above a test compound can be assayed by determining its ability to diminish the amount of labelled peptide or polypeptide which binds to the immobilized GST-fusion polypeptide. This may be determined by fractionating the glutathione-agarose beads by SDS-polyacrylamide gel electrophoresis.

Alternatively, the beads may be rinsed to remove unbound protein and the amount of protein which has bound can be determined by counting the amount of label present in, for example, a suitable scintillation counter.

An assay according to the present invention may also take the form of an *in vivo* assay. The *in vivo* assay may be performed in a cell line such as a yeast strain or mammalian cell line in which the relevant polypeptides or peptides are expressed from one or more vectors introduced into the cell.

The ability of a test compound to modulate interaction between ATM and p53 may be determined using a so-called two-hybrid assay.

For example, a polypeptide or peptide containing a fragment of ATM or p53 as the case may be, or a peptidyl analogue or variant thereof as disclosed, may be fused to a DNA binding domain such as that of the yeast transcription factor GAL4. (A particularly preferred fragment of ATM may include or be the kinase domain or a fragment of the kinase domain.) The GAL4 transcription factor includes two functional domains. These domains are the DNA binding domain (GAL4DBD) and the GAL4 transcriptional activation domain (GAL4TAD). By fusing one polypeptide or peptide to one of those domains and another polypeptide or peptide to the respective counterpart, a functional GAL4 transcription factor is

restored only when two polypeptides or peptides of interest interact. Thus, interaction of the polypeptides or peptides may be measured by the use of a reporter gene probably linked to a GAL4 DNA binding site which is capable of activating transcription of said reporter gene. This assay format is described by Fields and Song, 1989, Nature 340; 245-246. This type of assay format can be used in both mammalian cells and in yeast. Other combinations of DNA binding domain and transcriptional activation domain are available in the art and may be preferred, such as the LexA DNA binding domain and the VP60 transcriptional activation domain.

When looking for peptides or other substances which interfere with interaction between a ATM polypeptide or peptide and p53 polypeptide or peptide, the ATM or p53 polypeptide or peptide may be employed as a fusion with (e.g.) the LexA DNA binding domain, and the counterpart p53 or ATM polypeptide or peptide as a fusion with (e.g.) VP60, and involves a third expression cassette, which may be on a separate expression vector, from which a peptide or a library of peptides of diverse and/or random sequence may be expressed. A reduction in reporter gene expression (e.g. in the case of  $\beta$ -galactosidase a weakening of the blue colour) results from the presence of a peptide which disrupts the ATM/p53 interaction, which interaction is required for transcriptional activation of the  $\beta$ -galactosidase gene. Where a test substance is not peptidyl and may not be expressed from encoding nucleic acid within a said third expression cassette, a similar system may be employed with the test substance supplied exogenously.

When performing a two hybrid assay to look for substances which interfere with the interaction between two polypeptides or peptides it may be preferred to use mammalian cells instead of yeast cells. The same

principles apply and appropriate methods are well known to those skilled in the art.

In preferred assays according to the present invention,  
5 the end-point of the assay, that is to say that which is determined in order to assess the effect of the test agent on the interaction of interest, is phosphorylation of p53 or a fragment, variant or derivative thereof, or other molecule including a phosphorylation site  
10 homologous to one of those in p53 phosphorylated by ATM.

Thus, a further aspect of the present invention provides an assay method including

(a) bringing into contact a substance which  
15 includes at least a fragment of ATM which phosphorylates p53, a substance which includes at least a fragment of p53 including a site phosphorylated by ATM, and a test compound; and,

(b) determining phosphorylation at said site.

20 Of course, any suitable variant or derivative of ATM and/or p53 may be employed in such an assay.

Phosphorylation may be determined for example by immobilising p53 or a fragment, variant or derivative  
25 thereof, e.g. on a bead or plate, and detecting phosphorylation using an antibody or other binding molecule (such as Mdm2 or a fragment thereof) which binds the relevant site of phosphorylation with a different affinity when the site is phosphorylated from when the  
30 site is not phosphorylated. Such antibodies may be obtained by means of any standard technique as discussed elsewhere herein, e.g. using a phosphorylated peptide (such as a fragment of p53). Binding of a binding molecule which discriminates between the phosphorylated  
35 and non-phosphorylated form of p53 or relevant fragment, variant or derivative thereof may be assessed using any technique available to those skilled in the art, which



may involve determination of the presence of a suitable label, such as fluorescence. Phosphorylation may be determined by immobilisation of p53 or a fragment, variant or derivative thereof, on a suitable substrate such as a bead or plate, wherein the substrate is impregnated with scintillant, such as in a standard scintillation proximity assay, with phosphorylation being determined via measurement of the incorporation of radioactive phosphate. Phosphate incorporation into p53 or a fragment, variant or derivative thereof, may be determined by precipitation with acid, such as trichloroacetic acid, and collection of the precipitate on a nitrocellulose filter paper, followed by measurement of incorporation of radiolabeled phosphate.

An agent able to inhibit phosphorylation of p53 by ATM may include an ATP analogue or other substance able to affect the catalytic properties of the enzymically active site of ATM. An inhibitor of phosphorylation may interact with ATM within the kinase domain marked (for human ATM) in Figure 6. Residues within this domain are involved with interaction with p53 and catalysis of the phosphorylation. Residues outside of the domain may also be involved in interacting with p53 and agents which interfere with such interaction may affect the phosphorylation as discussed elsewhere herein.

The amount of test substance or compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.001 nM to 1mM or more concentrations of putative inhibitor compound may be used, for example from 0.01 nM to 100 $\mu$ M, e.g. 0.1 to 50  $\mu$ M, such as about 10  $\mu$ M. Greater concentrations may be used when a peptide is the test substance. Even a molecule which has a weak effect may be a useful lead compound for further investigation and development.

Compounds which may be used may be natural or synthetic chemical compounds used in drug screening programmes. Extracts of plants which contain several characterised or uncharacterised components may also be used.

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Antibodies directed to the site of interaction in either protein form a further class of putative inhibitor compounds. Candidate inhibitor antibodies may be characterised and their binding regions determined to provide single chain antibodies and fragments thereof which are responsible for disrupting the interaction.

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Antibodies may be obtained using techniques which are standard in the art. Methods of producing antibodies include immunising a mammal (e.g. mouse, rat, rabbit, horse, goat, sheep or monkey) with the protein or a fragment thereof. Antibodies may be obtained from immunised animals using any of a variety of techniques known in the art, and screened, preferably using binding of antibody to antigen of interest. For instance, Western blotting techniques or immunoprecipitation may be used (Armitage et al., 1992, Nature 357: 80-82). Isolation of antibodies and/or antibody-producing cells from an animal may be accompanied by a step of sacrificing the animal.

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As an alternative or supplement to immunising a mammal with a peptide, an antibody specific for a protein may be obtained from a recombinantly produced library of expressed immunoglobulin variable domains, e.g. using lambda bacteriophage or filamentous bacteriophage which display functional immunoglobulin binding domains on their surfaces; for instance see WO92/01047. The library may be naive, that is constructed from sequences obtained from an organism which has not been immunised with any of the proteins (or fragments), or may be one constructed using sequences obtained from an organism which has been

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exposed to the antigen of interest.

Antibodies according to the present invention may be modified in a number of ways. Indeed the term "antibody" should be construed as covering any binding substance having a binding domain with the required specificity. Thus the invention covers antibody fragments, derivatives, functional equivalents and homologues of antibodies, including synthetic molecules and molecules whose shape mimicks that of an antibody enabling it to bind an antigen or epitope.

Example antibody fragments, capable of binding an antigen or other binding partner are the Fab fragment consisting of the VL, VH, C1 and CH1 domains; the Fd fragment consisting of the VH and CH1 domains; the Fv fragment consisting of the VL and VH domains of a single arm of an antibody; the dAb fragment which consists of a VH domain; isolated CDR regions and F(ab')<sub>2</sub> fragments, a bivalent fragment including two Fab fragments linked by a disulphide bridge at the hinge region. Single chain Fv fragments are also included.

A hybridoma producing a monoclonal antibody according to the present invention may be subject to genetic mutation or other changes. It will further be understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of an antibody to the constant regions, or constant regions plus framework regions, of a different immunoglobulin. See, for instance, EP184187A, GB 2188638A or EP-A-0239400. Cloning and expression of chimeric antibodies are

described in EP-A-0120694 and EP-A-0125023.

Hybridomas capable of producing antibody with desired binding characteristics are within the scope of the present invention, as are host cells, eukaryotic or prokaryotic, containing nucleic acid encoding antibodies (including antibody fragments) and capable of their expression. The invention also provides methods of production of the antibodies including growing a cell capable of producing the antibody under conditions in which the antibody is produced, and preferably secreted.

The reactivities of antibodies on a sample may be determined by any appropriate means. Tagging with individual reporter molecules is one possibility. The reporter molecules may directly or indirectly generate detectable, and preferably measurable, signals. The linkage of reporter molecules may be directly or indirectly, covalently, e.g. via a peptide bond or non-covalently. Linkage via a peptide bond may be as a result of recombinant expression of a gene fusion encoding antibody and reporter molecule. The mode of determining binding is not a feature of the present invention and those skilled in the art are able to choose a suitable mode according to their preference and general knowledge.

Antibodies may also be used in purifying and/or isolating a polypeptide or peptide according to the present invention, for instance following production of the polypeptide or peptide by expression from encoding nucleic acid therefor. Antibodies may be useful in a therapeutic context (which may include prophylaxis) to disrupt the ATM/p53 (or ATR/p53) interaction with a view to inhibiting their activity. Antibodies can for instance be micro-injected into cells, e.g. at a tumour site, subject to radio- and/or chemo-therapy (as

discussed already above). Antibodies may be employed in accordance with the present invention for other therapeutic and non-therapeutic purposes which are discussed elsewhere herein.

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Other candidate inhibitor compounds may be based on modelling the 3-dimensional structure of a polypeptide or peptide fragment and using rational drug design to provide potential inhibitor compounds with particular molecular shape, size and charge characteristics.

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A compound found to have the ability to affect ATM and/or p53 activity has therapeutic and other potential in a number of contexts, as discussed. For therapeutic treatment such a compound may be used in combination with any other active substance, e.g. for anti-tumour therapy another anti-tumour compound or therapy, such as radiotherapy or chemotherapy. In such a case, the assay of the invention, when conducted *in vivo*, need not measure the degree of modulation of interaction between p53 and ATM (or appropriate fragment, variant or derivative thereof) or of modulation of p53 phosphorylation or activity caused by the compound being tested. Instead the effect on DNA repair, homologous recombination, cell viability, cell killing (e.g. in the presence and absence of radio- and/or chemo-therapy), retroviral integration, and so on, may be measured. It may be that such a modified assay is run in parallel with or subsequent to the main assay of the invention in order to confirm that any such effect is as a result of the inhibition of interaction between ATM and p53 caused by said inhibitor compound and not merely a general toxic effect.

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Thus, an agent identified using one or more primary screens (e.g. in a cell-free system) as having ability to interact with ATM and/or p53 and/or modulate activity of

ATM and/or p53 may be assessed further using one or more secondary screens. A secondary screen may involve testing for cellular radiosensitisation and/or sensitisation to radiomimetic drugs, effect on chromosome telomere length, inducing or preventing cell-cycle arrest following irradiation or other cellular insult, an effect of p53 induction following ionising radiation or other cellular insult, or induction of p21 or other downstream p53 target.

Following identification of a substance or agent which modulates or affects ATM and/or p53 activity, the substance or agent may be investigated further. Furthermore, it may be manufactured and/or used in preparation, i.e. manufacture or formulation, of a composition such as a medicament, pharmaceutical composition or drug. These may be administered to individuals, e.g. for any of the purposes discussed elsewhere herein.

As noted, the agent may be peptidyl, e.g. a peptide which includes a sequence as recited above, or may be a functional analogue of such a peptide.

As used herein, the expression "functional analogue" relates to peptide variants or organic compounds having the same functional activity as the peptide in question, which may interfere with the interaction between ATM and p53. Examples of such analogues include chemical compounds which are modelled to resemble the three dimensional structure of the ATM or p53 domain in the contact area, and in particular the arrangement of the key amino acid residues as they appear in ATM or p53.

In a further aspect, the present invention provides the use of the above substances in methods of designing or screening for mimetics of the substances.

Accordingly, the present invention provides a method of designing mimetics of ATM or p53 having the biological activity of p53 or ATM binding or inhibition, the activity of allosteric inhibition of p53 or ATM and/or the activity of modulating, e.g. inhibiting, ATM/p53 interaction, said method comprising:

(i) analysing a substance having the biological activity to determine the amino acid residues essential and important for the activity to define a pharmacophore; and,

(ii) modelling the pharmacophore to design and/or screen candidate mimetics having the biological activity.

Suitable modelling techniques are known in the art. This includes the design of so-called "mimetics" which involves the study of the functional interactions fluorogenic oligonucleotide the molecules and the design of compounds which contain functional groups arranged in such a manner that they could reproduced those interactions.

The designing of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. This might be desirable where the active compound is difficult or expensive to synthesise or where it is unsuitable for a particular method of administration, e.g. peptides are not well suited as active agents for oral compositions as they tend to be quickly degraded by proteases in the alimentary canal. Mimetic design, synthesis and testing may be used to avoid randomly screening large number of molecules for a target property.

There are several steps commonly taken in the design of a mimetic from a compound having a given target property. Firstly, the particular parts of the compound that are critical and/or important in determining the target

property are determined. In the case of a peptide, this can be done by systematically varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues constituting the active region of the compound are known as its "pharmacophore".

Once the pharmacophore has been found, its structure is modelled to according its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a variant of this approach, the three-dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner change conformation on binding, allowing the model to take account of this the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted on to it can conveniently be selected so that the mimetic is easy to synthesise, is likely to be pharmacologically acceptable, and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.



The mimetic or mimetics found by this approach can then be screened to see whether they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing.

Mimetics of this type together with their use in therapy form a further aspect of the invention.

The present invention further provides the use of a peptide which includes a sequence as disclosed, or a derivative, active portion, analogue, variant or mimetic, thereof able to interact with ATM or p53 and/or modulate, e.g. inhibit, interaction between ATM and p53 and/or modulate, e.g. inhibit, ATM and/or p53 activity, in screening for a substance able to interact with p53 and/or ATM, and/or modulate, e.g. inhibit, interaction between ATM and p53, and/or inhibit ATM and/or p53 activity.

Generally, such a substance, e.g. inhibitor, according to the present invention is provided in an isolated and/or purified form, i.e. substantially pure. This may include being in a composition where it represents at least about 90% active ingredient, more preferably at least about 95%, more preferably at least about 98%. Such a composition may, however, include inert carrier materials or other pharmaceutically and physiologically acceptable excipients. As noted below, a composition according to the present invention may include in addition to an inhibitor compound as disclosed, one or more other molecules of therapeutic use, such as an anti-tumour agent.

The present invention extends in various aspects not only to a substance identified as a modulator of ATM and p53 interaction and/or ATM or p53-mediated activity, property

or pathway, in accordance with what is disclosed herein, but also a pharmaceutical composition, medicament, drug or other composition comprising such a substance, a method comprising administration of such a composition to a patient, e.g. for a purpose discussed elsewhere herein, which may include preventative treatment, use of such a substance in manufacture of a composition for administration, e.g. for a purpose discussed elsewhere herein, and a method of making a pharmaceutical composition comprising admixing such a substance with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

A substance according to the present invention such as an inhibitor of ATM and p53 interaction may be provided for use in a method of treatment of the human or animal body by therapy which affects an ATM or p53-mediated activity in cells, e.g. tumour cells. Other purposes of a method of treatment employing a substance in accordance with the present invention are discussed elsewhere herein.

Thus, the invention further provides a method of modulating an ATM and/or p53-mediated activity, e.g. for a purpose discussed elsewhere herein, which includes administering an agent which modulates, inhibits or blocks the interaction of ATM with p53 protein, such a method being useful in treatment where such modulation, inhibition or blocking is desirable, or an agent which increase, potentiates or strengthens interaction of ATM with p53, useful in treatment where this is desirable.

The invention further provides a method of treatment which includes administering to a patient an agent which interferes with the interaction of ATM with p53. Exemplary purposes of such treatment are discussed elsewhere herein.

Whether it is a polypeptide, antibody, peptide, nucleic acid molecule, small molecule, mimetic or other pharmaceutically useful compound according to the present invention that is to be given to an individual, administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Pharmaceutical compositions according to the present invention, and for use in accordance with the present invention, may include, in addition to active ingredient, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material will depend on the route of administration, which may be oral, or by injection, e.g. cutaneous, subcutaneous or intravenous.

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil.

Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

5 For intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of  
10 relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be  
15 included, as required.

Liposomes, particularly cationic liposomes, may be used in carrier formulations.

20 Examples of techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.

25 The agent may be administered in a localised manner to a tumour site or other desired site or may be delivered in a manner in which it targets tumour or other cells.

30 Targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons, for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to  
35 enter the target cells.

Instead of administering these agents directly, they may

be produced in the target cells by expression from an encoding gene introduced into the cells, eg in a viral vector (a variant of the VDEPT technique - see below). The vector may targeted to the specific cells to be  
5 treated, or it may contain regulatory elements which are switched on more or less selectively by the target cells.

The agent (e.g. small molecule, mimetic) may be administered in a precursor form, for conversion to the  
10 active form by an activating agent produced in, or targeted to, the cells to be treated. This type of approach is sometimes known as ADEPT or VDEPT, the former involving targeting the activator to the cells by conjugation to a cell-specific antibody, while the latter  
15 involves producing the activator, e.g. an enzyme, in a vector by expression from encoding DNA in a viral vector (see for example, EP-A-415731 and WO 90/07936).

An agent may be administered in a form which is inactive  
20 but which is converted to an active form in the body. For instance, the agent may be phosphorylated (e.g. to improve solubility) with the phosphate being cleaved to provide an active form of the agent in the body.

25 A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated, such as cancer, virus infection or any other condition in which a ATM or p53-mediated effect is desirable.

30 Nucleic acid according to the present invention, encoding a polypeptide or peptide able to modulate, e.g. interfere with, ATM and p53 interaction and/or induce or modulate activity or other ATM or p53-mediated cellular pathway or  
35 function, may be used in methods of gene therapy, for instance in treatment of individuals, e.g. with the aim of preventing or curing (wholly or partially) a disorder

or for another purpose as discussed elsewhere herein.

Vectors such as viral vectors have been used in the prior art to introduce nucleic acid into a wide variety of different target cells. Typically the vectors are exposed to the target cells so that transfection can take place in a sufficient proportion of the cells to provide a useful therapeutic or prophylactic effect from the expression of the desired polypeptide. The transfected nucleic acid may be permanently incorporated into the genome of each of the targeted cells, providing long lasting effect, or alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are known in the art, see US Patent No. 5,252,479 and WO 93/07282. In particular, a number of viruses have been used as gene transfer vectors, including papovaviruses, such as SV40, vaccinia virus, herpesviruses, including HSV and EBV, and retroviruses. Many gene therapy protocols in the prior art have used disabled murine retroviruses.

As an alternative to the use of viral vectors other known methods of introducing nucleic acid into cells includes electroporation, calcium phosphate co-precipitation, mechanical techniques such as microinjection, transfer mediated by liposomes and direct DNA uptake and receptor-mediated DNA transfer.

Receptor-mediated gene transfer, in which the nucleic acid is linked to a protein ligand via polylysine, with the ligand being specific for a receptor present on the surface of the target cells, is an example of a technique for specifically targeting nucleic acid to particular cells.

A polypeptide, peptide or other substance able to modulate or interfere with the interaction of the relevant polypeptide, peptide or other substance as disclosed herein, or a nucleic acid molecule encoding a peptidyl  
5 such molecule, may be provided in a kit, e.g. sealed in a suitable container which protects its contents from the external environment. Such a kit may include instructions for use.

10 In further aspects the present invention provides for the provision of purified ATM and purified ATR. Purified ATM or ATR, for instance about 10% pure, more preferably about 20% pure, more preferably about 30% pure, more preferably about 40% pure, more preferably about 50%  
15 pure, more preferably about 60% pure, more preferably about 70% pure, more preferably about 80% pure, more preferably about 90% pure, more preferably about 95% pure, or substantially pure ATM or ATR is obtainable using DNA. Such DNA may be in any form which ATM or ATR  
20 bind, including single-stranded DNA, double-stranded DNA, nicked DNA, covalently closed DNA circles and so on. It is surprising that any and all of these are bound by ATM as shown experimentally below.

25 In one aspect the present invention provides the use of DNA for purifying ATM or ATR.

In another aspect the present invention provides a method of purifying ATM or ATR, the method including contacting  
30 ATM or ATR with DNA. A mixture of material including ATM or ATR may be contacted against immobilised DNA (e.g. on a bead or agarose, and either covalently or non-covalently such as via a specific binding molecule such as streptavidin or biotin) and molecules which do not  
35 bind washed off.

We have also established that ATM and ATR may be purified

using NTA, preferably in the presence of  $\text{Ni}^{2+}$ . The NTA may be on any suitable support such as agarose or sepharose. Thus, a further aspect of the present invention provides the use of NTA, preferably with  $\text{Ni}^{2+}$ , for purifying ATM or ATR.

Another aspect of the present invention provides a method of purifying ATM or ATR which includes, contacting ATM or ATR with NTA, preferably with  $\text{Ni}^{2+}$  and washing off molecules which do not bind.

Purification using DNA may be combined with purification using NTA, preferably with  $\text{Ni}^{2+}$ , sequentially or simultaneously.

Either technique may be used for identification of co-factors of ATM which modulate ATM activity, such as factors which affect the interaction between ATM and DNA.

The ATM contacted by DNA and/or NTA in a purification may be in a mixture of molecules, such as a cellular extract, such as from a cell of an A-T patient, a normal cell of an organism such as a human or a recombinant host cell expressing the protein from encoding DNA, such as a bacterial, eukaryotic (e.g. mammalian or yeast) or insect cell, such as in a baculovirus expression system. Purification may follow production of ATM recombinantly in a suitable expression system, such as a cell, by expression from encoding nucleic acid.

Following purification, ATM may be used as desired, e.g. in an assay for an agent which modulates its phosphorylation of p53 or other molecule, in raising or obtaining a specific antibody or other binding molecule, or in a therapeutic context such as to compensate in an individual for the absence of wild-type ATM (as in, for example, a patient with A-T).



Various further aspects and embodiments of the present invention will be apparent to those skilled in the art in view of the present disclosure. Certain aspects and embodiments of the invention will now be illustrated by way of example and with reference to the figures discussed already above.

#### *ATM binds to DNA*

A biotinylated random ds 50-mer oligonucleotide was coupled to streptavidin iron-oxide particles and these were employed to recover DNA binding proteins from HeLa cell nuclear extracts. This approach revealed that ATM interacts with particles bearing this random piece of ds DNA (Figure 1A). This binding is due to the presence of DNA, since streptavidin iron-oxide particles alone are unable to bind ATM (Figure 1A). Importantly, the sequence specific DNA-binding protein Sp1 and the non-specific DNA interacting protein complex containing RNA polymerase II (Pol II) are both unable to interact stably with the random DNA fragment employed in these studies (Figure 1A). Furthermore, DNA-PK<sub>cs</sub> present in the crude nuclear extract binds only very inefficiently to the immobilised DNA despite the fact that its DNA-targeting component Ku is present (data not shown). Notably, protein quantification reveals that, under conditions in which over 90% of ATM binds to the DNA-coupled particles, less than 2% of total nuclear protein is retained. Hence, the retention of ATM by DNA in these studies is highly specific.

The above assay revealed that ATM, or an ATM complex, is capable of binding to a random piece of duplex DNA. Additional studies revealed that ATM is also retained by particles containing another unrelated oligonucleotide, suggesting strongly that the interaction is not sequence-specific (data not shown). To investigate the DNA binding properties of ATM further, we tested a series of

DNAs with a variety of sizes and architectures. In these studies, binding and initial washes were conducted in the presence of 50 mM KCl, then bound material was eluted by sequential washes at 100, 250 and 500 nM KCl. Figure 1B demonstrates that the interaction between DNA and ATM is dependent on the size of the DNA-duplex. Thus, with a ds 15-mer, some ATM is still present in the unbound fraction and most bound material elutes in the lower salt wash. However, as the duplex size is increased, it becomes progressively more effective at binding ATM, such that when ds oligonucleotides of 50 bp or larger are employed, binding of ATM is almost quantitative and all bound ATM elutes in the higher salt wash (Figure 1B).

Since a variety of DNA structures are known to be produced by IR and are present during DNA-repair processes, we assessed the ability of ATM to bind to various types of DNA structure. Thus, assays were conducted employing particles coupled to a ds 100-mer oligonucleotide bearing a nick, a single-strand to double-strand transition, a gap of 35 bp, or a 10 base insertion loop. Notably, under the assay conditions employed, ATM binds to these DNA molecules with equal efficiency and apparent affinity as it does to the fully ds DNA oligonucleotide (Figure 1C). Additional studies show that ATM also binds effectively to ss DNA (Figure 1C) and that, as with ds-DNA, this binding is dependent on oligonucleotide length (data not shown). Furthermore, ATM binding in such experiments is competed effectively by linear and circular plasmid DNA, suggesting that DNA termini are not required for ATM binding (NDL, unpublished data). Taken together, these data show that ATM, or a complex containing this factor, is capable of interacting with DNA molecules containing a variety of different structures in an apparently non-sequence specific fashion. Our results also show that ATM prefers to bind to linear DNA, preferentially binding to the ends

of the DNA.

#### *Purification of ATM*

To increase our understanding of ATM further, we decided to attempt to purify this protein to essential homogeneity and thus separate it from other DNA-binding proteins, DNA repair factors, and protein and lipid kinases. The purification strategy we developed is outlined in Figure 2A. Since ATM is expressed ubiquitously and is located primarily in the cell nucleus, HeLa cell nuclear extract was used as starting material. Because no biochemical assay was available for ATM protein function, we monitored its purification by Western blot analysis using antibodies raised against two different portions of the protein (Lakin et al., 1996). This approach not only revealed the fractionation of ATM but also allowed us to pool fractions that were devoid of the abundant DNA-PK enzyme through simultaneously testing for the presence of DNA-PK<sub>CS</sub> and Ku. In light of the DNA-binding properties of ATM, we employed a final DNA affinity step in the purification scheme (Figure 2B, lane 4). Silver staining demonstrates that this leads to an essentially homogenous preparation of a ~350 kDa polypeptide, and Western blotting studies reveal that this is recognised strongly by ATM antiserum ATM.B (Figure 2B). Since this protein is also recognised by two other antibodies raised to distinct regions of the ATM polypeptide (data not shown), we conclude that the purified protein is indeed ATM. As revealed in Figure 2B, whilst ATM is enriched throughout the purification procedure, Ku, DNA-PK<sub>CS</sub>, and the abundant ss DNA binding protein Replication Protein A (RPA) are all efficiently removed. Quantitative Western blotting and silver-staining reveal that the final yield of ATM is approximately 25% and indicate that ATM is of relatively low abundance, comprising around 0.002% of total nuclear protein by weight.

Purified ATM possesses an associated p53 kinase activity. Notably, as for DNA-PK (Hartley et al., 1995), purified ATM preparations were found to be devoid of detectable kinase activity towards PI and a variety of phosphorylated PI derivatives. Although we cannot exclude that ATM phosphorylates these or related phospholipids under certain conditions or in the presence of additional components, we conclude that ATM is not a lipid kinase. To assay for possible ATM-associated protein kinase activity, we performed *in vitro* kinase assays using equivalent amounts of various recombinant or purified proteins that we speculated may be ATM substrates. Certain candidate substrates, such as DNA-PK<sub>cs</sub>, Ku, proliferating cellular nuclear antigen (PCNA), and the 34 kDa subunit of RPA (RPA-p34), were chosen by virtue of their association with DNA damage detection and/or involvement in DNA repair. We also tested Sp1 and p53, since these are both good substrates for DNA-PK and because A-T cells display aberrant induction of p53 in response to IR. A final protein tested was I $\kappa$ B, since recent data have implicated this is an ATM target (Jung et al., 1995; Jung et al., 1997). Given that we had found that ATM binds to DNA, we included a DNA oligonucleotide known to activate DNA-PK in all initial kinase reactions.

Notably, none of DNA-PK<sub>cs</sub>, RPA-p45 and PCNA was phosphorylated efficiently by purified ATM (Figure 3A). However, longer exposures of autoradiograms reveals weak phosphorylation of both the 70 kDa subunit of Ku (Ku70) and Sp1 by ATM preparations (data not shown). Furthermore, prolonged exposures also reveal that ATM is capable of autophosphorylation (data not shown), consistent with previous rough studies employing ATM that had been immunoprecipitated directly from crude cell extracts (Keegan et al., 1996) (likely to contain all sorts of impurities). Most significantly, however,

several independently purified ATM preparations were consistently found to phosphorylate p53 with high efficiency (Figure 3A) (contrary to the mentioned results of Keegan et al.). Taken together, these data reveal that, under our assay conditions, a protein kinase activity co-purifies with ATM that phosphorylates p53 efficiently, and Sp1 and Ku70 weakly. Importantly, DNA-PK efficiently phosphorylates p53, Sp1, Ku70 and RPA-p34 in vitro, revealing that the ATM-associated kinase activity exhibits a different substrate specificity from that of DNA-PK. This, together with the absence of detectable DNA-PK<sub>cs</sub> or Ku in our ATM preparations argues strongly against the possibility that the ATM-associated protein kinase activity is imparted by DNA-PK contamination.

Although the above results reveal that a p53 kinase activity co-purifies with ATM, prolonged silver staining reveals additional polypeptides in our ATM preparations (data now shown). The possibility therefore existed that the p53 kinase activity that we had detected was not mediated by ATM but by a contaminating protein. To address this issue, we immunoprecipitated ATM from purified ATM preparations using polyclonal antibodies raised against either the N-terminal region (ATM.N) or an internal region (ATM.B) of the ATM polypeptide (Lakin et al., 1996). After washing the immunoprecipitated material extensively in the presence of 500 mM KCl and 0.1% Nonidet-P40, it was employed in kinase reactions using p53 as substrate. To establish the purity of the immunoprecipitated material, purified ATM was biotinylated and immunoprecipitated in parallel with ATM employed in the kinase reactions. The biotinylated precipitated proteins were then visualised by Western transfer and probing with streptavidin conjugated horseradish peroxidase.

As illustrated in Figure 3B, a biotinylated protein of approximately 350 kDa in size, the predicted molecular mass of ATM, is precipitated in these studies by anti-ATM antisera but not by pre-immune sera. Notably, no other proteins are consistently precipitated by both ATM antisera in these assays (a polypeptide of ~100 kDa is apparent in the ATM.N precipitation in Figure 3B but is not present in ATM.B immunoprecipitates and was not consistently observed in subsequent experiments using ATM.N).

Most importantly, these experiments revealed that p53 kinase activity is immunoprecipitated by the two ATM antisera. Greater ATM associated kinase activity is observed with ATM.N than with ATM.B, despite only slightly higher amounts of ATM being precipitated by ATM.N (Figure 3C). One possible explanation for this is that ATM.B, which recognises epitopes close to the ATM kinase domain, impairs ATM protein kinase activity. These studies show that the p53 kinase activity present in our ATM preparations follows ATM through a further highly stringent immuno-affinity purification step, and suggest strongly that ATM directly mediates p53 phosphorylation. Although unlikely in our opinion, it remains a possibility that p53 is phosphorylated by a distinct polypeptide that has escaped our detection methods and which remains associated with ATM throughout the stringent purification and immunoprecipitation protocols employed.

#### *ATM associated kinase activity is stimulated by DNA*

Given that ATM can interact with DNA, we investigated whether ATM associated protein kinase activity is stimulated by a nucleic acid cofactor. To achieve this, we performed *in vitro* kinase assays using purified ATM either in the absence or presence of increasing amounts of DNA. Because previous studies have revealed that co-

localisation of DNA-PK and Sp1 to the same DNA molecule increases phosphorylation efficiency (Lees-Miller et al., 1992; Gottlieb and Jackson, 1993), we employed a linear plasmid molecule bearing multiple p53 binding sites.

5 These studies revealed that DNA addition leads to marked stimulation of p53 phosphorylation by DNA-PK (Figure 4A, middle). Strikingly DNA addition was also found to result in marked stimulation of p53 phosphorylation in reactions containing ATM (Figure 4A, top). Thus, purified ATM preparations contain a DNA-stimulatable p53 kinase activity. Longer exposures of autoradiograms reveal that the ATM polypeptide is also subject to phosphorylation in such assays and that this phosphorylation is stimulated by DNA (data not shown).

10 Experiments employing equimolar amounts of DNA-PK and ATM revealed that the stimulation of p53 kinase activity by DNA is similar for ATM and DNA-PK, and that the stoichiometry of p53 phosphorylation by ATM is at least as high as that catalysed by DNA-PK (data not shown).

15 Although DNA-dependent kinase activity was consistently observed in ATM preparations, the degree of activation was variable. In this regard, additional polypeptides were apparent in several preparations that displayed high levels of DNA activatability. Thus, it is possible that co-purifying polypeptides may be involved in high level ATM DNA dependent kinase activity. Notably, DNA-PK and ATM preparations both displayed significant but low levels of p53 kinase activity in the absence of DNA. It is not currently known, however, whether this reflects bona fide DNA-independent phosphorylation or results from small amounts of DNA in the protein preparations.

20 Parallel experiments using cyclin A/cdk2 demonstrate no increase of p53 phosphorylation upon DNA addition (Figure 4A), and a variety of other protein kinases that we have tested are not stimulated by DNA. These results therefore show that increased protein phosphorylation is not a general effect of adding DNA to p53 kinase assays

and reveal that ATM is highly unusual in its ability to be stimulated by DNA.

We had established that ATM binds to various types of linear DNA molecule (see Figure 1). Our binding competition studies indicated that ATM also interacts with supercoiled and nicked DNA (data not shown). We tested whether ATM associated kinase activity is affected differentially by various DNA structures. p53 kinase assays were performed in the absence of DNA or in the presence of increasing amounts of either supercoiled or restriction enzyme-linearised plasmid DNA.

Notably, ATM is activated by supercoiled and linear DNA (Figure 4B), and additional studies revealed that good activation also occurs with nicked plasmid DNA molecules (data not shown). By contrast, DNA-PK is stimulated strongly by linear but only weakly by supercoiled plasmid DNA (Figure 4B; based on previous studies, the weak activation by the latter probably reflects small amounts of nicked and/or linear DNA in the supercoiled plasmid preparation). These results are therefore consistent with data showing that ATM is able to interact with many different types of DNA structure. Furthermore, they show that, although ATM is analogous to DNA-PK in that its associated kinase activity is stimulated by DNA, the DNA cofactor requirements of the two enzymes are different.

*ATM associated kinase activity phosphorylates p53 at two sites*

To determine the site(s) of p53 that are phosphorylated by ATM, bacterially expressed p53 was radioactively phosphorylated by ATM in either the presence or absence of DNA. Labelled p53 was purified by electrophoresis, digested by trypsin, and the resulting products separated by reverse-phase HPLC. Analysis of the resulting radioactive profiles showed a major peak eluting at 11-12% acetonitrile. A novel set of radioactive p53 derived



HPLC polypeptide peaks, which elute at 28-29% acetonitrile were induced substantially in the presence of DNA. Phosphoamino acid analysis revealed that the DNA induced peaks contained peptides labelled at both serine and threonine residues, suggesting either two distinctly labelled co-eluting peptides, or a single peptide containing both phosphoserine and phosphothreonine residues (data not shown). Radioactive peaks with similar elution properties were identified following phosphorylation of p53 by DNA-PK (Figure 5B) or casein kinase I (data not shown). Previous studies have revealed that both DNA-PK and casein kinase I phosphorylate the N-terminal region of p53 (Lees-Miller et al., 1992; Milne et al., 1992). Initial attempts to sequence p53-derived peaks were unsuccessful, presumably because they possess blocked amino-termini. However, cleavage with endoproteinase Asp-N allowed sequencing of each. Notably, release of counts at cycles 9 and 12 of Edman degradation of peptide 2a reveals that the sites of phosphorylation correspond to p53 residues Ser-15 and Thr-18. Ser-15 has previously been demonstrated to be a phosphorylation site for DNA-PK (Lees-Miller et al., 1992). However, no detectable DNA-PK exists in our ATM preparations (see above).

We therefore conclude that a novel DNA dependent kinase activity is associated with ATM that targets Ser-15 and Thr-18 of p53.

*DNA-PK ATR has an associated kinase activity that phosphorylates p53 at Ser15 and Thr18*

Given the fact than an activity in our ATM preparations was found to phosphorylate residue Thr18 of p53, we decided to test whether DNA-PK is also able to phosphorylate this site. To this end, p53 was incubated in the presence of radiolabelled [ $\alpha^{32}\text{P}$ ] ATP with purified human DNA-PK (a preparation consisting of the Ku and DNA-

PKcs components of the enzyme; prepared as described in Hartley et al., 1995) in either the absence or presence of a linearised plasmid DNA molecule, then, as described for analysis of ATM-mediated phosphorylation events, the p53 was treated with protease to generate phospho-peptides and these were analysed by reverse-phase HPLC. These studies revealed that, as in the ATM studies, a set of related peptides eluting at around 28-29% acetonitrile (co-fractionating with ATM-derived peptides, 2a, b, and c; compare figures 5B and D) were phosphorylated by a DNA-PK associated kinase activity in a DNA-inducible fashion. Furthermore, analysis of these revealed that they correspond to p53 peptides containing phosphorylation on residues Ser15 and Thr19 (Figure 5B). Subsequent studies using antibodies that recognise specifically p53 that is phosphorylated on Ser15 or Thr19 (see below for details of antibody preparation) confirmed that the DNA-PK-associated kinase activity phosphorylates both of these residues of p53. Therefore, contrary to expectations, DNA-PK-associated kinase activity phosphorylates p53 on Thr18 as well as Ser15.

*ATR has an associated kinase activity that phosphorylates p53 at Ser15*

Given that both DNA-PK-associated and a ATM-associated kinase activities phosphorylate p53 on Ser15 and Thr18, we decided to see whether other kinases exist that can target these residues. To facilitate this approach, we generated rabbit polyclonal antibodies that specifically recognise p53 that is phosphorylated on Thr18 (they do not recognise unphosphorylated p53 nor p53 that is phosphorylated solely on Ser15 nor is phosphorylated elsewhere). Similarly, we generated rabbit polyclonal antibodies that specifically recognised p53 phosphorylated on Ser15. These antibodies were generated by immunising rabbits with specific p53-based phospho-peptides (containing either Thr18 or Ser15

phosphorylated), then preparing the antibodies with the desired recognition characteristics (those that recognised the specific phosphorylated peptides but not unphosphorylated versions of these peptides) by chromatography on columns bearing immobilised unphosphorylated peptide and columns bearing specific phosphorylated peptides.

To assess kinases activated in human cell extracts capable of phosphorylating p53 on Ser15, HeLa nuclear extract was fractionated chromatographically (see below) then the resulting fractions were incubated with full-length p53 protein and non-radioactively labelled ATP, either in the absence or presence of DNA. Phosphorylation of p53 was then assessed by subjecting the samples to SDS-polyacrylamide gel electrophoresis and Western immunoblotting. As shown in Figure 10, two main peaks of kinase activity (termed "activity 1" and "activity 2") capable to targeting Ser15 (S15) were detected in fractions of HeLa nuclear extract that had been chromatographed on Q-sepharose. Further analysis of these fractions revealed that both activities were stimulated by DNA. Furthermore, Western blotting revealed that fractions comprising "activity 1" contained the ATM-related protein ATR, whereas those comprising "activity 2" contained DNA-PKcs (Figure 10). In addition, other experiments revealed a third weaker, activity peak in fractions between those comprising activity 1 and activity 2, which corresponded to ATM. Further purification of activity peak 2 revealed that it corresponded to DNA-PK. Further fractionation of activity 1 revealed that, under all chromatographic separation techniques utilised, the DNA-activated p53 Ser15 kinase activity co-eluted with ATR. Indeed, through following this kinase activity, ATR could be purified to near homogeneity (e.g. Figure 11; ATR was the only polypeptide whose elution was found to consistently

parallel that of the kinase activity). Thus, in addition to DNA-PK and ATM targeting p53 Ser15, we have made the surprising discovery that this residue is also phosphorylated by a kinase activity associated with ATR.

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*Effect of p53 phosphorylation on interaction with Mdm2*

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To test whether phosphorylation on Ser15 or Thr18 of p53 affects its interaction with Mdm-2, phosphorylated and unphosphorylated p53-derived peptides were generated and were assessed for Mdm-2 binding by ELISA analysis. The four peptides used contained p53 residues 11 to 25 (in the sequence NH<sub>2</sub>-SGSGEPPLSOETFSDLWKL-COOH; where the underlined sequence is that derived from p53) that were unphosphorylated (1); phosphorylated on residue equivalent to p53 residue Ser15(2); phosphorylated on residue equivalent to p53 residue Thr18(3); or phosphorylated on two residues, equivalent to p53 residue Ser15 and Thr18(4). Binding of Mdm-2 derivatives occurred effectively with unphosphorylated peptide 1 but was found to be inhibited dramatically in the cases of peptides 3 and 4, which contained phosphorylated Thr18. In contrast, binding was only impaired slightly by phosphorylation on Ser15 (peptide 2). We therefore conclude that phosphorylation on Thr18 of p53 has a dramatic effect on its interaction with Mdm-2 and that phosphorylation of this site is likely to play a key role in regulating p53 responses in vivo.

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*Additional purification method for ATM*

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HeLa nuclear extract was applied to Ni<sup>2+</sup> - NTA agarose (Qiagen). We found that ATM binds very tightly to this matrix, but not very well to Ni<sup>2+</sup> - IDA matrices.

35

5 ml of nuclear extract was loaded onto a 1 x 2.5 cm column of Ni<sup>2+</sup> - NTA agarose in the following buffer (Buffer D; 25 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10% Glycerol, 1 mM MgCl<sub>2</sub>, 20 mM imidazole). The column was

washed extensively (10 column volumes) before applying a linear gradient of 20 mM - 500 mM imidazole in buffer D. Virtually pure ATM (as judged by silver stain analysis of 8% polyacrylamide gels) eluted near the end of the imidazole gradient. Less pure fractions of ATM eluted at the start of the gradient.

This provides a purification strategy for ATM or ATR that may be used alone, or in combination with various other chromatographic steps, e.g. DNA affinity chromatography as discussed already above.

#### *Discussion*

We have demonstrated that ATM is retained on immobilised particles bearing DNA molecules. Notably, ATM binds to both ds and ss DNA in vitro, and studies employing a variety of unrelated oligonucleotides provide indication that this interaction is not sequence dependent. By exploiting these and other biochemical properties of ATM, we have developed a strategy to purify this polypeptide from HeLa nuclear extracts to near homogeneity. The high purity of our final ATM preparations and the fact that ATM in such preparations can re-bind to DNA provides indication that ATM interacts with DNA directly. Although this appears somewhat different from the situation with DNA-PKcs, which requires Ku to associate stably with DNA under our assay conditions, UV protein-DNA cross-linking has revealed that, in the context of the DNA-PKcs/Ku holoenzyme, DNA-PKcs does make close contacts with DNA (Gottlieb and Jackson, 1993). DNA-PKcs and ATM may interact with DNA through similar mechanisms.

Because the C-terminal region of ATM possesses homology to the catalytic domain of mammalian PI 3-kinase, it has been speculated that ATM may phosphorylate inositol phospholipids. However, despite conducting lipid phosphorylation assays under various conditions and with

a variety of potential substrates, no ATM-associated lipid kinase activity was detected in our ATM preparations. These data are thus consistent with recent studies demonstrating that ATM-containing immunoprecipitates possess no detectable lipid kinase activity (Jung et al., 1997). Although we cannot discount the possibility that ATM modifies particular PI derivatives under certain conditions or in association with additional cofactors, we tentatively conclude that, as has been proposed for DNA-PKcs (Hartley et al., 1995) and FRAP (Brown et al., 1995), ATM is not a lipid kinase.

In contrast, our purified ATM preparations consistently possess protein serine/threonine kinase activity.

Recently (Keegan et al., 1996) have performed rough experiments which might suggest that ATM-containing immunoprecipitates phosphorylate an ~350 kDa polypeptide, suggesting that ATM can modify itself (though the preparations would have contained all sorts of impurities, including kinases). We observe that purified ATM preparations are capable of some degree of ATM auto-phosphorylation.

In addition, we have tested ATM for its ability to modify a variety of other polypeptides. Notably, despite the fact that I $\kappa$ B has been implicated as an ATM target by in vivo functional studies (Jung et al., 1995) and has recently been reported to be phosphorylated by ATM-containing immunoprecipitates (Jung et al., 1997), under our assay conditions we do not detect significant I $\kappa$ B phosphorylation by ATM. Although alternatives exist, one explanation for this discrepancy is that I $\kappa$ B phosphorylation detected in the studies of (Jung et al., 1997) was mediated by a co-immunoprecipitating factor that is separated from ATM during our purification scheme.

Another protein that has been implicated as a possible ATM target by virtue of defective regulation in A-T cells is RPA (Liu and Weaver, 1993; Cheng et al., 1996).

However, we have been unable to detect significant phosphorylation of RPA by ATM, suggesting that ATM regulates RPA indirectly. In contrast to the above, we observe low but detectable phosphorylation of Sp1 and the 70 kDa subunit of Ku by ATM. Although the significance of these phosphorylation events is uncertain, these findings raise the interesting possibilities that ATM plays a role in regulating Sp1-dependent transcription and controlling the activity of the Ku/DNA-PKcs holoenzyme.

By far the most efficient substrate for ATM that we have identified, however, is p53. Importantly, the p53 kinase activity we have detected consistently co-purifies with ATM, elutes from the final DNA affinity purification step with the same profile as the ATM polypeptide itself, and further co-purifies with ATM through an additional stringent immunoprecipitation procedure. These data provide strong indication that p53 kinase activity is an inherent property of the ATM polypeptide.

In a manner strikingly reminiscent of the activation of DNA-PK by DNA strand breaks and ds to ss DNA transitions, we find that ATM and ATR associated p53 kinase activity is stimulated markedly by the addition of a DNA cofactor. There are several reasons why this DNA-stimulated protein kinase activity is unlikely to be mediated by contaminating DNA-PK. First, titration studies reveal that, to provide the observed level of p53 phosphorylation, the DNA-PKcs content of ATM and ATR preparations would have to be essentially as great as that of ATM itself. Clearly, this is not the case - silver staining and Western blotting reveal that, if any residual DNA-PK does exist in our most purified ATM and ATR preparations, it is present at levels undetectable by

the methods employed in this study. Second, the substrate specificity observed in ATM and ATR preparations is distinct from that of DNA-PK. Third, whereas ATM and ATR-associated kinase activity is stimulated similarly by supercoiled and linear plasmid molecules, DNA-PK is only activated strongly by the latter.

There are several possible ways in which ATM and ATR might be stimulated by DNA, and each of these may contribute to the effects that we observe. (The mechanism of action provides no limitation to the nature and scope of the present invention.)

One possibility is that DNA binding by ATM and ATR activates the catalytic potential of the proteins directly. Another is that the co-localisation of ATM and ATR and its target DNA binding protein on the same DNA molecule serves to potentiate interactions between the kinase and its target. In line with one or both of the above models, we have observed that ATM auto-phosphorylation is also enhanced by DNA, albeit to a lesser degree than that observed with p53.

Alternatively, at least part of the dramatic stimulation of p53 phosphorylation upon DNA addition could be explained by the binding of p53 to DNA inducing a conformational change in p53 that makes it a more effective ATM or ATR substrate. Thus, Ser-15 and Thr-18 might only become accessible to ATM after p53 is bound to DNA. In accordance with such a model, it is known that the conformation of p53 does change upon binding to DNA (Halazonetis et al., 1993), and it has been observed that several naturally occurring p53 mutants that are defective in sequence-specific DNA binding exhibit reduced phosphorylation at Ser-15 (Ullrich et al., 1993).

Given the DNA-PK paradigm, and because of the previously



described role of ATM in DNA damage signalling, it might be tempting to speculate that ATM or ATR protein kinase activity in vivo is triggered by specific types of DNA damage or stalled DNA replication forks that occur in response to IR. However, unlike DNA-PK, which is activated strongly in vitro only by DNA molecules bearing perturbations in the DNA double-helix, we find that ATM interacts with all types of DNA structure that we have tested. It is, therefore, possible that ATM is active constitutively in mammalian cells. An alternative model, which we currently favour, is that ATM and ATR associate with other polypeptides rather like DNA-PKcs interacts with Ku, and it is the function of these additional components to restrict ATM or ATR activity under normal circumstances and only allow their activation after exposure to DNA damaging agents. In this regard, it is interesting to note that yeast genetic data indicate the *S. cerevisiae* and *S. pombe* homologues of ATM or ATR function in conjunction with other polypeptides in DNA damage signalling (reviewed in Elledge, 1996; Carr, 1997), and that biochemical studies reveal that ATM exists as a large complex of ~2 MDa in crude cell extracts (GCMS, unpublished data).

Together with genetic data indicating that ATM functions upstream of p53 in a pathway for signalling IR-induced DNA damage, our findings provide indication that, following genomic insult, ATM and ATR phosphorylate p53 directly. Such a model would help to explain the deficient up-regulation of p53 in response to IR in A-T cells and this, in turn, would explain at least some of the cell cycle checkpoint control defects of A-T cells. Interestingly, recent studies indicate that ATM interacts with p53 directly (Watters et al., 1997) providing a possible mechanism for optimising the efficiency of ATM-mediated p53 phosphorylation in the cellular context. Indeed, since p53 itself binds to DNA strand breaks and

DNA insertion loops (Balkalkin et al., 1994; Lee et al., 1995; Reed et al., 1995), p53 could actually play a role in targeting ATM or ATR to sites of DNA damage. Such a model is particularly attractive when one considers that Ser-15 and Thr-18 reside in conserved and functionally important regions of the p53 polypeptide. Moreover, Ser-15 of p53 has been shown to be phosphorylated in vivo (reviewed in Anderson and Lees-Miller, 1992; Steegenga et al., 1996). In addition, although Thr-18 has not yet been identified as a physiological site for p53 modification, it is noteworthy that this residue is highly conserved in p53, and that around 8% of p53 phosphorylation in vivo occurs at Thr residues (Samad et al., 1986). In light of these points, it will clearly be of great interest to analyse the phosphorylation status of p53 Ser-15 and Thr-18 in wild-type and A-T cells, and to determine their degree of phosphorylation in response to IR.

Interestingly, phosphorylation of the N-terminal region of p53 has been proposed to effect both the stability and the transcriptional activation potential of p53 (reviewed in Ko and Prives, 1996; Steegenga et al., 1996). Indeed, mutation of Ser-15 impairs the capacity of p53 to prevent S-phase progression and affects p53 stability (Fiscella et al., 1993). Furthermore, p53 mutants unable to activate transcription show reduced phosphorylation at this site (Ullrich et al., 1993). Although no experiments have investigated the role of Thr-18 in p53 function directly, it is noteworthy that this residue forms part of the minimal p53 binding site for Mdm2, which functions as a negative regulator of p53 function (Oliner et al., 1993). Significantly, Mdm-2 binding has been linked both to repressing p53-dependent transcriptional activation and targeting p53 for degradation within the cell (Momand et al., 1992; Oliner et al., 1993; Kubbutat et al., 1997). An attractive scenario, therefore, is that phosphorylation of p53 by ATM or ATR may inhibit Mdm2

interaction, thus both stabilising p53 and de-repressing its transcriptional activity. Consistent with this, we find that the binding of p53-derived peptides to Mdm2 is strongly inhibited by phosphorylation of Thr18.

It is emphasized that suggested mechanisms of action and models for ATM and p53 function discussed above are presented without limitation to the nature and scope of the present invention.

#### EXPERIMENTAL PROCEDURES

##### *DNA interaction studies*

Oligonucleotides: one DNA strand containing a 5' biotin group (indicated by a "B" below) was annealed with complementary oligonucleotide(s) and bound to streptavidin-coated iron-oxide particles (Dynabeads; Dynal, Oslo, Norway). HeLa nuclear extract, or ATM enriched extract (Q-Sepharose pool; see below) was incubated on ice for 30 min. with the DNA-iron oxide particles. After washing with 5 x 0.5 ml of D\* Buffer (25 mM HEPES-KOH, pH 7.6, 20% glycerol, 2 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1mM Na Metabisulfite) containing 50 mM KCl, protein was eluted with 500 mM KCl D\* buffer or in gradual stepwise manner with KCl concentrations of 100 mM, 250 mM and 500 mM in buffer D\*. Fractions were analysed for ATM protein content by Western blotting using a previously described rabbit polyclonal antisera raised against amino acid residues 1980-2337 of ATM (Lakin et al., 1996).

##### *Oligonucleotides:*

ds 15-mer:	5' B-CCTGCCCTTGCCTGA-3'
	5' TCAGGCAAGGGCAGG-3'
ds 25-mer	5' B-CCTGCCCTTGCCTGACGCTATTAGT-3'
	5' ACTAATAGCGTCAGGCAAGGGCAGG-3'
ds 50-mer	

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5' B-TTGTAACGACGGCCAGTGAATTCATCATCAATAATATACCTTATTTTG-  
3'

5' CAAAATAAGGTATATTATTGATGATGAATTCAGTGGCCGTCGTTTACAA-3'  
ds 75-mer

5  
5'  
BGATCGAATCCGATAGAGTATAGATAGAGTAAAGTTTAAATACTTATATAGATAG  
AGTATAGATAGAGGGTTCAAA-3'

5' TTTGAACCCTCTATCTATACTCTATCTATATAAGTATTTAACTTTACTC  
TATCTATACTCTATCGGATTCGATC-3'

10 ss 50-mer

5' B-TTGTAACGACGGCCAGTGAATTCATCATCAATAATATACCTTATTTTG-  
3'

15 For the following, a biotinylated 100-mer oligonucleotide  
(DYNO) was used as a "backbone" to which other  
oligonucleotides were annealed.

DYNO 5' B-

20 CCTGCCCTTGCCCTGACGCTATTAGTTCATCTATTTGTTTTGCTAATTCGA  
TTGGAATCGAAACGGTCACATATTCTTTTTTGAAGTATTCCTCGGCATA-3'

nicked oligo, DYNO + DAM2 + DAM3: ds/ss transition, DYNO  
+ DAM3; gapped ds oligo, DYNO + DAM3 + DAM5; 10 bp  
insertion, DYNO + DAM6.

25

DAM2:

5' TATGCCGAGGAAATCAGTCAAAAAGAATATGTGACCGTTTCGATTCCAA-3'

DAM3:

30 5' TCGAATTAGCAAAACAAATAGATGAACTAATAGCGTCAGGCAAGGGCAGG-3'

DAM5: 5' TATGCCGAGGAAATC-3'

DAM6:

35

5'  
TATGCCGAGGAAATCAGTCAAAAAGAATATGTGACCGTTTCGAATTAGCAAAAC  
AAATAGATGAACTAATAGCGTCAGGCAAGGGCAGG-3'

*ATM purification*

All steps were performed at 4°C. HeLa nuclear extract (20 ml) was applied to a Q-Sepharose column (35 ml, 1.5 x 20 cm) equilibrated in D\* buffer (25 mM HEPES-KOH, pH 7.6, 20% glycerol, 2 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM Na Metabisulfite) containing 50 mM KCl. After washing with 2 column vol. of 50 mM KCl D\*, protein was eluted with a continuous salt gradient of 50 mM - 500 mM KCl in D\* buffer. ATM eluted between 160 and 200 mM KCl. Fractions containing ATM and devoid of DNA-PK (as judged by Western blot analysis) were pooled and, after diluting to 100 mM KCl in D\* buffer, were loaded onto a heparin agarose column (1.5 x 6 cm) pre-equilibrated in 100 mM KCl D\* buffer. The column was washed with 2 column vol. of 100 mM KCl D\* buffer before eluting with a continuous gradient of 50 mM - 500 mM KCl in buffer D\*. ATM was again followed by Western blot analysis and eluted between 200 and 220 mM KCl. Peak fractions were pooled and dialysed against 50 mM buffer D\*. Peak ATM fractions were then incubated with gentle mixing for 1 h. with 200 µg biotinylated 50 bp ds DNA conjugated to streptavidin iron-oxide particles. Unbound protein was rebound to fresh DNA-iron oxide particles. Particles were collected via a magnet and were washed 5 x with 0.5 ml of 50 mM KCl D\* buffer before eluting ATM with 2 x 75 µl 500 mM KCl buffer D\*. Purified ATM was snap-frozen and stored at -70°C.

*ATR purification*

ATR purification was carried out as set out in the description.

*Immunological Methods*

Western immunoblot analysis was performed as previously (Lakin et al., 1996). Sp1 antisera were purchased from Serotec Ltd. (Oxford, UK). RPA-p70 and RNA polymerase II antisera were also utilised. Phospho-specific antisera

were generated as described herein.

Immunoprecipitations were performed by incubating biotinylated or untreated purified ATM in parallel with serum for 1 h. on ice in D\* buffer containing 50 mM KCl. Protein A Sepharose was added and the reaction incubated with slow rotation for a further h. at 4°C. Beads were washed at high stringency seven times in 500 µl of D\* buffer containing 500 mM KCl and 0.1% NP-40. Biotinylated immunoprecipitated proteins were visualised by 7% SDS-PAGE followed by Western blotting and probing with streptavidin-conjugated horse-radish peroxidase. Un-biotinylated immunoprecipitated proteins were washed a further two times in 500 µl 1 x Z' buffer prior to addition to kinase reactions (see below).

#### *Phosphorylation assays*

Kinase reactions were performed in 20 µl containing: 10 µl Z' buffer (25 mM HEPES-KOH pH 7.9, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 20% glycerol, 0.1% NP-40, 1mM DTT); 11 fmol ATM, DNA-PK or cyclin A/cdk2; 50-100 ng substrate and 0-30 fmol of DNA. Reactions were assembled and incubated for 3 min. on ice prior to addition of 10 µCi [γ-<sup>32</sup>P] ATP and incubation at 30°C for 15 min. Phosphorylated proteins were subjected to 7% SDS-PAGE and visualised by autoradiography.

#### *Mapping of p53 phosphorylation sites*

Recombinant p53 (10-20 pmol; purified as previously (Hupp et al., 1992)) was incubated with 12-24 ng of purified ATM or DNA-PK in the presence of 100 µM ATP containing 10<sup>6</sup>-10<sup>7</sup> cpm/nmol [<sup>32</sup>P]-γATP under reaction conditions described above. Linearised (pG<sub>13</sub>-CAT) or supercoiled (pBS-SK; Stratagene, USA) DNA were included in DNA-PK and ATM reactions, respectively, where indicated. After 30 min. at 30°C, reactions were terminated by transferring to an ice water bath. Following TCA precipitation,

labelled p53 was resolved by 10% SDS-PAGE and visualised by autoradiography. The gel section containing labelled p53 was excised and the protein eluted and TCA precipitated as described (Alessi et al., 1996). The washed TCA pellet was either digested directly with alkylated trypsin (Promega, Southampton, UK) or, for ASP-N digestion, solubilised first in 0.2% v/v Triton X-100 and digested overnight with 1:5 w:w Asp-N (Boehringer Mannheim) and, where indicated, followed by overnight digestion with trypsin. The supernatant containing digested protein was chromatographed on a Vydac 218TP54 C18 column (Separations Group, Hesperia, CA) equilibrated with 0.1% v/v trifluoroacetic acid (TFA), and eluted with a linear acetonitrile gradient. The flow rate was 0.8 ml/min. and 0.4 ml fractions were collected. Peak fractions were coupled covalently to a Sequelon acrylamide membrane and analysed on an Applied Biosystems 470A sequencer using the modified programme described by (Stokoe et al., 1992) to determine Edman degradation cycle numbers corresponding to radioactivity release.

#### *Additional Purification of ATM*

Purification using NTA has been described already above.

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Claims:

1. An assay method for a compound able to modulate the interaction between (i) ATM or a protein having an associated kinase activity and (ii) p53 or any protein having ATM phosphorylation sites homologous to those of p53, the method including the steps of:

(a) bringing into contact a substance including a peptide fragment of (i) or a derivative, variant or analogue thereof, a substance including the relevant fragment of p53 or any protein having phosphorylation sites homologous to p53, or a variant, derivative or analogue thereof, and a test compound; and

(b) determining interaction or binding between said substances and test compound.

2. An assay method for a compound able to modulate the interaction between (i) ATM or a protein having an associated kinase activity and (ii) p53 or any protein having ATM phosphorylation sites homologous to those of p53, the method including the steps of:

(a) bringing into contact a substance which includes at least a fragment of (i) which phosphorylates p53 or any protein having phosphorylation sites homologous to those of p53, a substance which includes at least a fragment of p53 or any protein, which includes a site which is phosphorylated by (i), and a test compound; and

(b) determining phosphorylation at said site.

3. An assay method for a compound able to affect p53 activity by modulation of interaction between (i) ATM or a protein having an associated kinase activity and (ii) p53, the method including the steps of:

(a) bringing into contact a substance which is p53 or a fragment, derivative, variant or analogue thereof, and a test compound; and

(b) determining p53 activity in the presence and

absence of (i)

4. The method according to any one of claims 1 to 3 wherein the protein having an associated kinase activity is DNA-PK or ATR.

5. An agent capable of modulating interaction between (i) ATM or a protein having an associated kinase activity and (ii) p53 or any protein having ATM phosphorylation sites homologous to p53 obtained using a method according to any of claims 1 to 4.

6. An agent according to claim 5 capable of modulating ATM-mediated phosphorylation on p53 or said homologous sites.

7. A peptide fragment of p53 capable of modulating interaction between ATM or a protein having an associated kinase activity and p53.

8. A peptide according to claim 7 capable of modulating phosphorylation of p53 by ATM or by a protein having an associated kinase activity.

9. A peptide according to claim 7 or claim 8 which has a sequence found in human p53 including Thr18.

10. A peptide according to claim 7 or claim 8 which has a sequence found in human p53 including Ser15.

11. A peptide according to any one of claims 7 to 10 wherein the protein having an associated kinase activity is DNA-PK or ATR.

12. A nucleic acid isolate encoding a peptide according to any of claims 7 to 11.

13. A peptide fragment of ATM or a protein having an associated kinase activity which is capable of modulating interaction between ATM or the protein and p53.

14. A peptide according to claim 13 capable of modulating phosphorylation of p53 by ATM or a protein having an associated kinase activity.

15. The peptide of claim 13 or claim 14 wherein the protein having an associated kinase activity is DNA-PK or ATR.

16. A nucleic acid isolate encoding a peptide according to any one of claims 13 to 15.

17. An agent or peptide fragment or nucleic acid isolate according to any of claims 5 to 16 for use in a method of treatment by therapy involving modulating ATM action.

18. Use of an agent or peptide fragment or nucleic acid isolate according to any of claims 5 to 17 in the manufacture of a medicament for modulating ATM action.

19. An assay method for a compound able to affect DNA binding by ATM or a protein having an associated kinase activity, the method including the steps of:

(a) bringing into contact a substance which is ATM or a protein having an associated kinase activity, or a fragment, variant or derivative which is able to bind DNA, DNA, and a test compound, under conditions wherein, in the absence of the test compound being an inhibitor of DNA binding by ATM or the protein having an associated kinase activity, said substance binds DNA; and

(b) determining binding between said substance and said DNA.

20. The assay method according to claim 19 wherein the

protein having an associated kinase activity is DNA-PK or ATR.

21. An agent capable of affecting DNA binding by ATM  
obtained using a method according to claim 19 or claim  
20.

22. An agent according to claim 21 for use in a method  
of treatment by therapy involving modulating ATM action.

23. Use of an agent according to claim 21 in the  
manufacture of a medicament for modulating ATM action.

24. A method of purifying ATM or ATR including  
contacting a mixture of molecules including ATM or ATR  
with DNA, washing off molecules which do not bind the  
DNA, and recovering ATM or ATR from the DNA-bound  
fraction.

25. Use of DNA for purifying ATM or ATR.

26. A method of purifying ATM or ATR, including  
contacting a mixture of molecules including ATM or ATR  
with NTA, washing off molecules which do not bind the  
NTA, and recovering ATM or ATR from the NTA-bound  
fraction.

27. A method according to claim 26 wherein the mixture  
is contacted with NTA in the presence of  $\text{Ni}^{2+}$ .

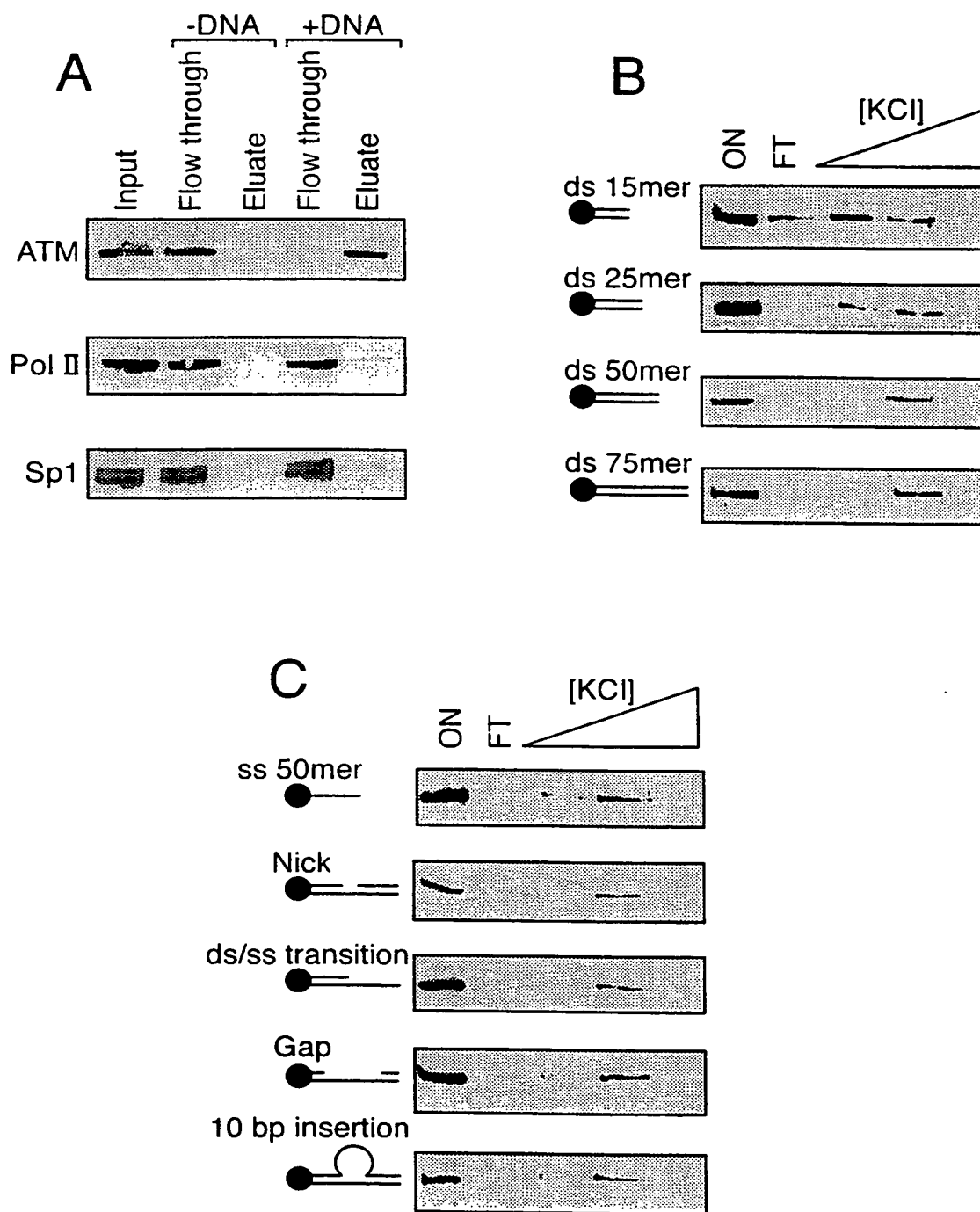
28. Substantially pure ATM.

29. Substantially pure ATR.

ADP  
B4

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Fig.1.







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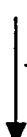
Fig.2.

**A**

HeLa Nuclear Extract



Q-sepharose



160-200 mM KCl

Heparin Agarose



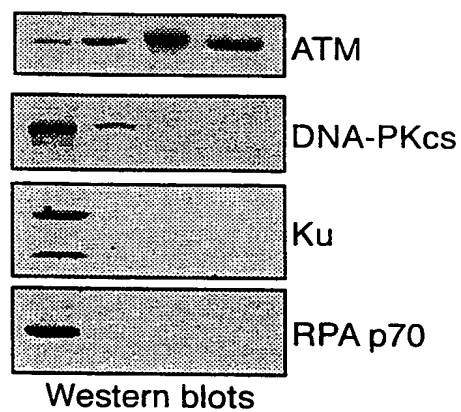
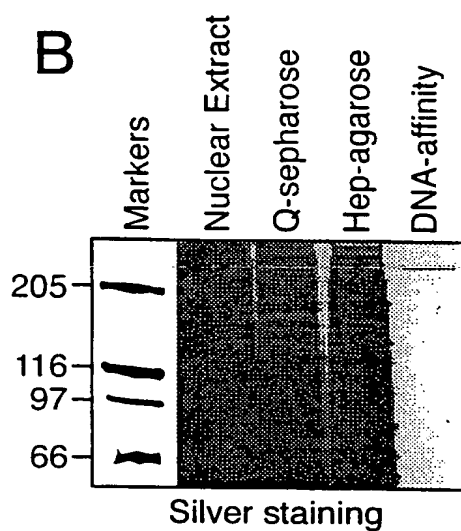
200-220 mM KCl

DNA-affinity



500 mM KCl

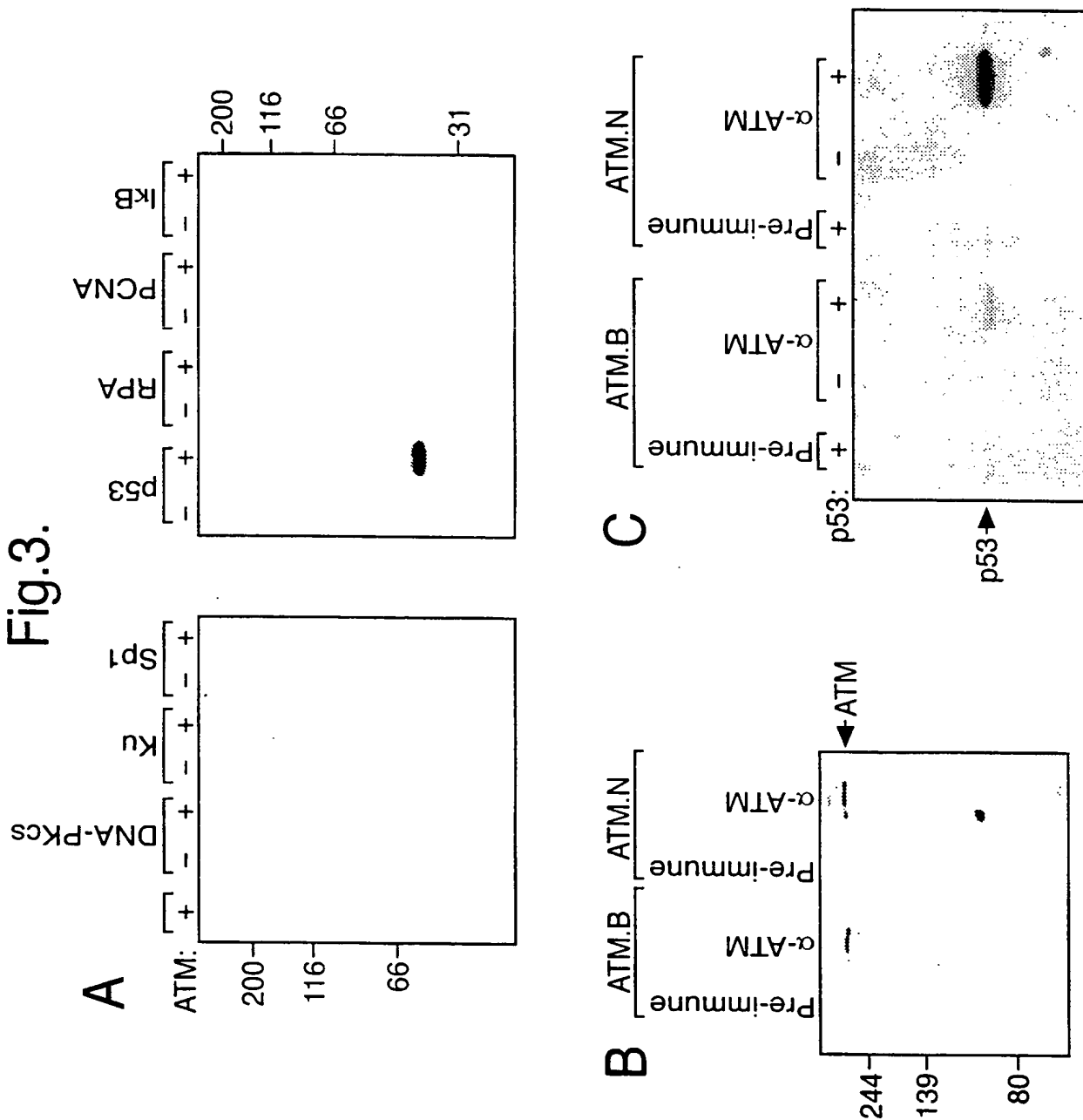
~Pure ATM

**B**



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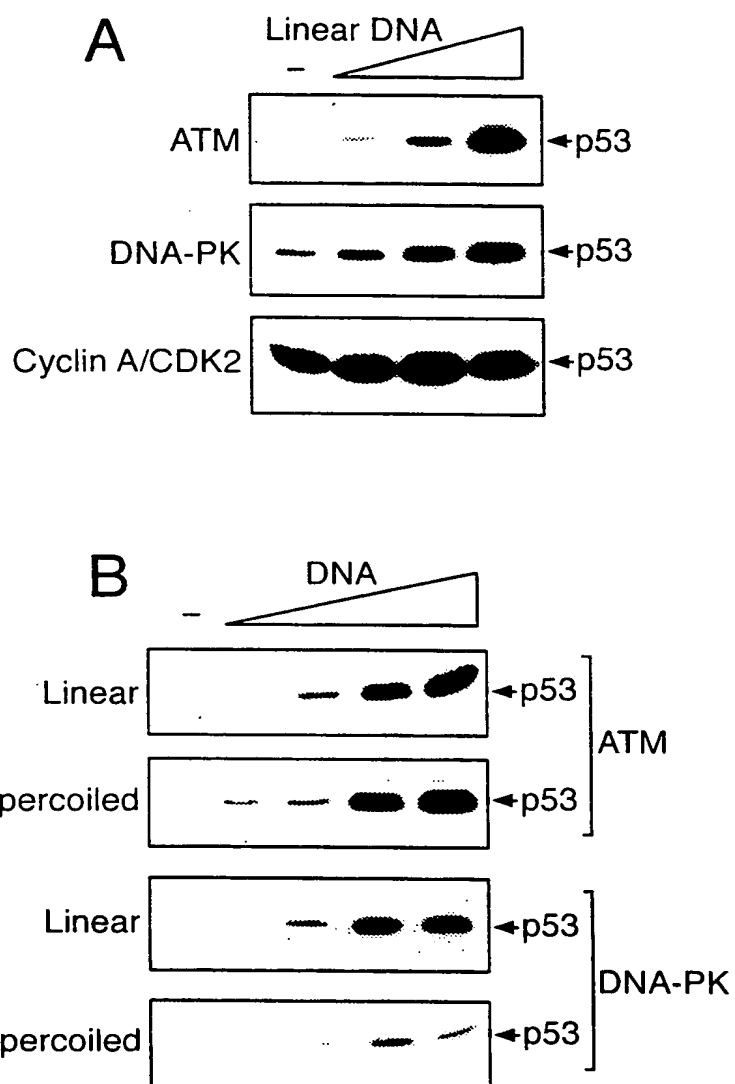
Fig.3.





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Fig.4.





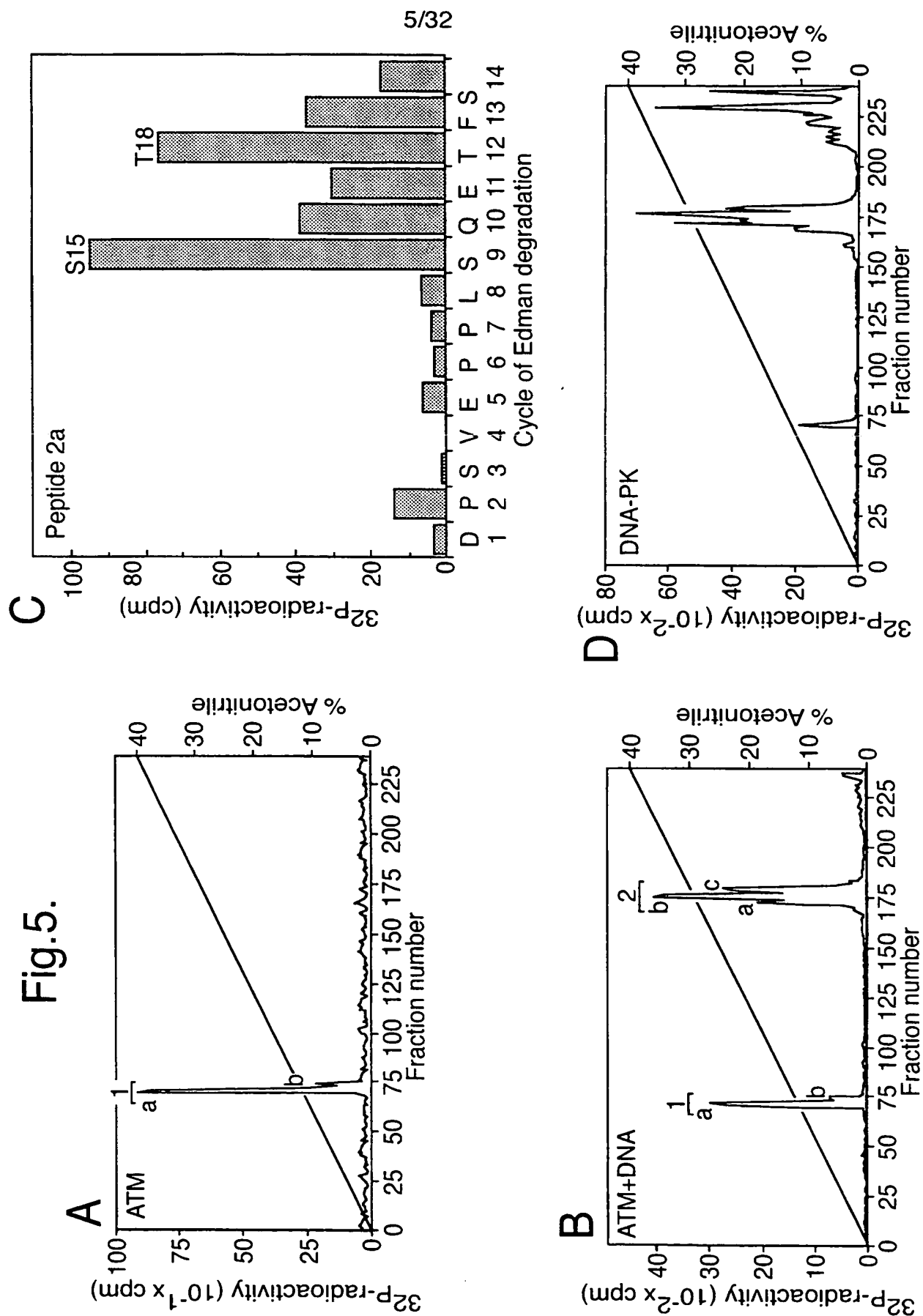






Figure 6a

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IKCANRRAPRLKCQELNLYIMDTVKDSSNGAIYGADCSNILLKDILSVRKYWCIEISQQQ
WLELFSVYFRLYLKPSQDVHRVLVARI IHAVTKGCCSQTDLNSKFLDFFSKAIQCARQ
EKSSSGLNHILAALTI FLKTLAVNFRIRVCELGDEILPTLLYIWTQHRLNDSLKEVITIE
LFQLQIYIHHPKGAKTQEKGAYESTKWSRILYNLYDLLVNEISHIGSRGKYSFGFRNTIA
VKENLIELMADICHQVFNEDTRSLEISQSYTTTQRESSDYSVPCKRKKIELGWEVIKDH
LQKSONDFDLVPWLQIATQLISKYPASLPNCELSPMLMILSQLLPQQRHGERTPYVLRC
LTEVALCQDKRSNLESSQKSDLLKLWNKIWCITFRGISSEQIQAEFGLGAI IQGSLV
EVDREFWKLFTGSACRPSPCAVCCLTALTTISIVPGAVKMGIEQNMCEVNRFSLSKESI
MKWLLFPYQLEGDLENSTEVPPILHSNFPHLVLEKILVSLTMKNCKAAMNFFQSVPCECH
HQKDKEELSFSEVEELFLQTTFDKMDFLTIVRECGIEKHQSSIGFSVHQNLESIDRCL
LGLSEQLLNHYSSEITNSETLVRCRLLVGVGLGCYCYMGVIAEEEEAYKSELFQKANSML
QCAGESITLTKNKTNEEFRIKSLRNMMLQCTRLSNCTKSPNKIASGFFLRLLTSKLM
NDIADIKSLASFICKPDRGEVESMEDDTNGNLMEVEDQSSMNLFNNDYDSSVSDANE
PGESQSTIGA INPLAEYLSKQDLLFLDMLKFLCLCVTTAQTNTVSFRAADIRKLLML
IDSSTLEPTKSLHLMYLMMLKELPGEEYPLPMEDVLELLKPLSNVCSLYRRDQDVCKT
ILNHVLHVVKNLGQSNMDSENTRDAQGQFLTVIGAFWHLTKERKIFSVRMALVNCLKT
LLEADPYSKWAILNVMGKDFPVNEVFTQFLADNHHQVRMLAAESINRLFQDTKGDSRL
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ICEKQALFALCKSVKENGLEPHLVKKVLEKVSETFGYRREDFMASHLDYLVLEWNLQ
DTEYNLSSFPFILLNYTNIEDFYRSCYKVLIPHLVIRSHFDEVKSIANQIQEDWKSLLT
DCFPKILVNILPYFAYEGTRDSGMAQQORETATKVYDMLKSENLLGKQIDHLFISNLPEI
VVELLMTLHEPANSSASQSTDLCDFSGDLDPAPNPPHPFPHVVIKATFAYISNCHTKLK
SILEILSKSPDSYQKILLAIQEAETNNVYKHKRILKIYHLFVSLLLKDKISGLGGAW
AFVLRDVIYTLIHYINQRPSCIMDVSLRSFSLCCDLLSQVCQTAVTYCKDALENHLHI
VGTILPLVYEQVEVQKQVLDLLKYLVIDNKNENLYITIKLLDPFDPHVVFKDLRITQQ
KIKYSRGPFSLLEEINHFSLSVSYDALPLTRLEGLKDLRRQLELHKDQMDIMRASQDN
PQDGIMVKLVVNLQLSKMAINHTGEKEVLEAVGSCLEGEVGPIDFSTIAIQHSDASYT
KALKLFEDKELQWTFIMLTYNLNTLVEDCVKVRSAAVTCLKNILATKTGHSFWEIYKMT
TDPMLAYLQPFRTSRKCFLEVPFRDKENPFEGLDINLWIPLENHDIWIKTLTCLFLD
SGGTKEIILQLLKPMCEVKTDFCQTVLPYLIHDILLQDTNESWRNLLSTHVQGFSTSC
RHFSQTSRSTTPANLDSSEHFFRCCLDKKSQRTMLAVVDYMRQRKRPSSGTIFNDAFW
LDLNYLEVAKVAQSCAAHFTALLYAEIYADKKSMDQEKRSIAFEEGSQSTTISLSEK
SKEETGISLQDLLLEIYRSIGEPDSLYGCGGKMLQPIITRLRTYEHEAMWGKALVTYDL
ETAIPSTRQAGIIQALQNLGLCHILSVYKGLDYENKDWCPLEELHYQAAWRNMQWD
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YPTLSRLQAIGELISIGELFSRSVTHRQLSEVYIKWQKHSQLLKDSDFSQEPIMALRT
VILEILMEKEMDNSQRECIKIDILTKHLVELSILARTFKNTQLPERAIFQIKQYNSVSG
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KAVENYINCLLSGEEHDMVFRCLSLWLENSGVSEVNGMKRDKMKIPTYKFLPLMYQL
AARMGTKMMGGLGFHEVLNLLISRISMDHPHHTLFILALANANRDEFLTKEVARRSR
ITKNVPKQSSQLDEDRTAANRIICTIRSRPQMVRSEALCDAYIILANLDAQWKTQ
RKGINIPADQPIITKLKNEEDVVVPTIMEIKVDHTGEYGNLVTIQSFKAEFRLAGGVNLPK
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VFMDVCQNFQPVFRYFCMEKFLDPAIWF EKRLAYTRSVATSSIVGYILGLGDRHVONIL
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Figure 6b (1)

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51 TTTGACCTTC CGAGTGCAGT GAGGCATACA TCACAATTTG GAATTATGCA
101 TTGGTTTATC AATTTACTTG TTTATTGTCA CCCTGCTGCC CAGATATGAC
151 TTCATGAGGA CAGTGATGTG TGTCTGAAA TTGTGAACCA TGAGTCTAGT

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Figure 6b (11)

201 ACTTAATGAT CTGCTTATCT GCTGCCGTCA ACTAGAACAT GATAGAGCTA  
251 CAGAACGAAA GAAAGAAGTT GAGAAATTTA AGCGCCTGAT TCGAGATCCT  
301 GAAACAATTA AACATCTAGA TCGGCATTCA GATTCCAAAC AAGGAAAATA  
351 TTTGAATTGG GATGCTGTTT TTAGATTTTT ACAGAAATAT ATTCAGAAAG  
401 AAACAGAATG TCTGAGAATA GCAAAACCAA ATGTATCAGC CTCAACACAA  
451 GCCTCCAGGC AGAAAAAGAT GCAGGAAATC AGTAGTTTGG TCAAATACTT  
501 CATCAAATGT GCAAAACAGAA GAGCACCTAG GCTAAAATGT CAAGAACTCT  
551 TAAATTATAT CATGGATACA GTGAAAGATT CATCTAATGG TGCTATTTAC  
601 GGAGCTGATT GTAGCAACAT ACTACTCAA GACATTCTTT CTGTGAGAAA  
651 ATACTGGTGT GAAATATCTC AGCAACAGTG GTTAGAATTG TTCTCTGTGT  
701 ACTTCAGGCT CTATCTGAAA CCTTCACAAG ATGTTCATAG AGTTTTAGTG  
751 GCTAGAATAA TTCATGCTGT TACCAAAGGA TGCTGTTCTC AGACTGACGG  
801 ATTAAATTCC AAATTTTTGG ACTTTTTTTC CAAGGCTATT CAGTGTGCGA  
851 GACAAGAAAA GAGCTCTTCA GGTCTAAATC ATATCTTAGC AGCTCTTACT  
901 ATCTTCCTCA AGACTTTGGC TGTCAACTTT CGAATTCGAG TGTGTGAATT  
951 AGGAGATGAA ATTCTTCCCA CTTTGCTTTA TATTTGGACT CAACATAGGC  
1001 TTAATGATTG TTTAAAAGAA GTCATTATTG AATTATTTCA ACTGCAAATT  
1051 TATATCCATC ATCCGAAAGG AGCCAAAACC CAAGAAAAAG GTGCTTATGA  
1101 ATCAACAAAA TGGAGAAGTA TTTTATACAA CTTATATGAT CTGCTAGTGA  
1151 ATGAGATAAG TCATATAGGA AGTAGAGGAA AGTATTCTTC AGGATTTCTG  
1201 AATATTGCCG TCAAAGAAAA TTTGATTGAA TTGATGGCAG ATATCTGTCA  
1251 CCAGGTTTTT AATGAAGATA CCAGATCCTT GGAGATTTCT CAATCTTACA  
1301 CTACTIONACA AAGAGAATCT AGTGATTACA GTGTCCCTTG CAAAAGGAAG  
1351 AAAATAGAAC TAGGCTGGGA AGTAATAAAA GATCACCTTC AGAAGTCACA  
1401 GAATGATTTT GATCTTGTGC CTTGGCTACA GATTGCAACC CAATTAATAT  
1451 CAAAGTATCC TGCAAGTTTA CCTAACTGTG AGCTGTCTCC ATTACTGATG  
1501 ATACTATCTC AGCTTCTACC CCAACAGCGA CATGGGGAAC GTACACCATA  
1551 TGTGTTACGA TGCCTTACGG AAGTTGCATT GTGTCAAGAC AAGAGGTCAA  
1601 ACCTAGAAAG CTCACAAAAG TCAGATTTAT TAAACTCTG GAATAAAATT  
1651 TGGTGTATTA CCTTTCTGTT TATAAGTTCT GAGCAAATAC AAGCTGAAAA  
1701 CTTTGGCTTA CTTGGAGCCA TAATTCAGGG TAGTTTAGTT GAGGTTGACA  
1751 GAGAATCTCT GAAGTTATTT ACTGGGTCAG CCTGCAGACC TTCATGTCCT  
1801 GCAGTATGCT GTTTGACTTT GGCAGTGACC ACCAGTATAG TTCCAGGAGC  
1851 GGTAAAAATG GGAATAGAGC AAAATATGTG TGAAGTAAAT AGAAGCTTTT



Figure 6 b (111)

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1901 CTTTAAAGGA ATCAATAATG AAATGGCTCT TATTCTATCA GTTAGAGGGT
1951 GACTTAGAAA ATAGCACAGA AGTGCCTCCA ATTCTTCACA GTAATTTTCC
2001 TCATCTTGTA CTGGAGAAAA TTCTTGTGAG TCTCACTATG AAAAACTGTA
2051 AAGCTGCAAT GAATTTTTC CAAAGCGTGC CAGAATGTGA ACACCACCAA
2101 AAAGATAAAG AAGAACTTTC ATTCTCAGAA GTAGAAGAAC TATTTCTTCA
2151 GACAACTTTT GACAAGATGG ACTTTTTAAC CATTGTGAGA GAATGTGGTA
2201 TAGAAAAGCA CCAGTCCAGT ATTGGCTTCT CTGTCCACCA GAATCTCAAG
2251 GAATCACTGG ATCGCTGTCT TCTGGGATTA TCAGAACAGC TTCTGAATAA
2301 TTAATCATCT GAGATTACAA ATTACAGAAAC TCTTGTCGG TGTTACGTC
2351 TTTTGGTGGG TGTCCTTGGC TGCTACTGTT ACATGGGTGT AATAGCTGAA
2401 GAGGAAGCAT ATAAGTCAGA ATTATTCCAG AAAGCCAACT CTCTAATGCA
2451 ATGTGCAGGA GAAAGTATCA CTCTGTTTAA AAATAAGACA AATGAGGAAT
2501 TCAGAAITGG TTCTTGAGA AATATGATGC AGCTATGTAC ACGTTGCTTG
2551 AGCAACTGTA CCAAGAAGAG TCCAAATAAG ATTGCATCTG GCTTTTTTCT
2601 GCGATTGTTA ACATCAAAGC TAATGAATGA CATTGCAGAT ATTTGTAAAA
2651 GTTTAGCATC CTTTCATCAA AAGCCATTTG ACCGTGGAGA AGTAGAATCA
2701 ATGGAAGATG ATACTAATGG AAATCTAATG GAGGTGGAGG ATCAGTCATC
2751 CATGAATCTA TTTAACGATT ACCCTGATAG TAGTGTTAGT GATGCAAACG
2801 AACCTGGAGA GAGCCAAAGT ACCATAGGTG CCATTAATCC TTTAGCTGAA
2851 GAATATCTGT CAAAGCAAGA TCTACTTTTC TTAGACATGC TCAAGTTCTT
2901 GTGTTTGTGT GTAACACTG CTCAGACCAA TACTGTGTCC TTTAGGGCAG
2951 CTGATATTCG GAGGAAATTG TTAATGTTAA TTGATTCTAG CACGCTAGAA
3001 CCTACCAAAT CCTCCACCT GCATATGTAT CTAATGCTTT TAAAGGAGCT
3051 TCCTGGAGAA GAGTACCCCT TGCCAATGGA AGATGTTCTT GAACTTCTGA
3101 AACCCTATC CAATGTGTGT TCTTTGTATC GTCGTGACCA AGATGTTTGT
3151 AAACTATTT TAAACCATGT CCTTCATGTA GTGAAAAACC TAGGTCAAAG
3201 CAATATGGAC TCTGAGAACA CAAGGGATGC TCAAGGACAG TTTCTTACAG
3251 TAATTGGAGC ATTTTGGCAT CTAACAAAGG AGAGGAAATA TATATTCTCT
3301 GTAAGAATGG CCTAGTAAA TTGCCTTAAA ACTTTGCTTG AGGCTGATCC
3351 TTATTCAAAA TGGGCCATT TTAATGTAAT GGGAAAAGAC TTTCTGTAA
3401 ATGAAGTATT TACACAATTT CTTGCTGACA ATCATCACC AGTTGCGATG
3451 TTGGCTGCAG AGTCAATCAA TAGATTGTT CAGGACACGA AGGGAGATTC
3501 TTCCAGGTTA CTGAAAGCAC TTCCTTGAA GCTTCAGCAA ACAGCTTTTG

```



Figure 6b (iv)

3551 AAAATGCATA CTTGAAAGCT CAGGAAGGAA TGAGAGAAAT GTCCCATAGT  
3601 GCTGAGAACC CTGAAACTTT GGATGAAATT TATAATAGAA AATCTGTTTT  
3651 ACTGACGTTG ATAGCTGTGG TTTTATCCTG TAGCCCTATC TGCGAAAAAC  
3701 AGGCTTTGTT TGCCCTGTGT AAATCTGTGA AAGAGAATGG ATTAGAACCT  
3751 CACCTTGTGA AAAAGGTTTT AGAGAAAGTT TCTGAAACTT TTGGATATAG  
3801 ACGTTTAGAA GACTTTATGG CATCTCATTT AGATTATCTG GTTTTGAAT  
3851 GGCTAAATCT TCAAGATACT GAATACAACT TATCTTCTTT TCCTTTTATT  
3901 TTATTAAACT ACACAAATAT TGAGGATTTT TATAGATCTT GTTATAAGGT  
3951 TTTGATTCCA CATCTGGTGA TTAGAAGTCA TTTTGATGAG GTGAAGTCCA  
4001 TTGCTAATCA GATTCAAGAG GACTGGAAAA GTCTTCTAAC AGACTGCTTT  
4051 CCAAAGATTC TTGTAAATAT TCTTCCTTAT TTTGCCTATG AGGGTACCAG  
4101 AGACAGTGGG ATGGCACAGC AAAGAGAGAC TGCTACCAAG GTCTATGATA  
4151 TGCTTAAAAG TGAAAACTTA TTGGGAAAAC AGATTGATCA CTTATTCAAT  
4201 AGTAATTTAC CAGAGATTGT GGTGGAGTTA TTGATGACGT TACATGAGCC  
4251 AGCAAATTCT AGTGCCAGTC AGAGCACTGA CCTCTGTGAC TTTTCAGGGG  
4301 ATTTGGATCC TGCTCCTAAT CCACCTCATT TTCCATCGCA TGTGATTAAA  
4351 GCAACATTTG CCTATATCAG CAATTGTCAT AAAACCAAGT TAAAAAGCAT  
4401 TTTAGAAATT CTTTCCAAAA GCCCTGATTC CTATCAGAAA ATTCTTCTTG  
4451 CCATATGTGA GCAAGCAGCT GAAACAAATA ATGTTTATAA GAAGCACAGA  
4501 ATTCTTAAAA TATATCACCT GTTTGTTAGT TTATTACTGA AAGATATAAA  
4551 AAGTGGCTTA GGAGGAGCTT GGGCCTTTGT TCTTCGAGAC GTTATTTATA  
4601 CTTTGATTCA CTATATCAAC CAAAGGCCTT CTTGTATCAT GGATGTGTCA  
4651 TTACGTAGCT TCTCCCTTTG TTGTGACTTA TTAAGTCAGG TTGCCCAGAC  
4701 AGCCGTGACT TACTGTAAGG ATGCTCTAGA AAACCATCTT CATGTTATTG  
4751 TTGGTACACT TATACCCCTT GTGTATGAGC AGGTGGAGGT TCAGAAACAG  
4801 GTATTGGACT TGTTGAAATA CTTAGTGATA GATAACAAGG ATAATGAAAA  
4851 CCTCTATATC ACGATTAAGC TTTTAGATCC TTTTCCTGAC CATGTTGTTT  
4901 TTAAGGATTT GCGTATTACT CAGCAAAAAA TCAAATACAG TAGAGGACCC  
4951 TTTTCACTCT TGGAGGAAAT TAACCATTTT CTCTCAGTAA GTGTTTATGA  
5001 TGCACTTCCA TTGACAAGAC TTGAAGGACT AAAGGATCTT CGAAGACAAC  
5051 TGGAECTACA TAAAGATCAG ATGGTGGACA TTATGAGAGC TTCTCAGGAT  
5101 AATCCGCAAG ATGGGATTAT GGTGAAACTA GTTGTCAATT TGTTCAGTT  
5151 ATCCAAGATG GCAATAAACC AACTGGTGA AAAAGAAGTT CTAGAGGCTG  
5201 TTGGAAGCTG CTTGGGAGAA GTGGGTCCTA TAGATTTCTC TACCATAGCT





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Figure 6b (v)

5251 ATACAACATA GTAAAGATGC ATCTTATACC AAGGCCCTTA AGTTATTTGA  
5301 AGATAAAGAA CTTCAAGTGA CCTTCATAAT GCTGACCTAC CTGAATAACA  
5351 CACTGGTAGA AGATTGTGTC AAAGTTCGAT CAGCAGCTGT TACCTGTTTG  
5401 AAAAAACATTT TAGCCACAAA GACTGGACAT AGTTTCTGGG AGATTTTATAA  
5451 GATGACAACA GATCCAATGC TGGCCTATCT ACAGCCTTTT AGAACATCAA  
5501 GAAAAAAGTT TTTAGAAGTA CCCAGATTTG ACAAAGAAAA CCCTTTTGAA  
5551 GGCCTGGATG ATATAAATCT GTGGATTCCCT CTAAGTGAAA ATCATGACAT  
5601 TTGGATAAAG ACACTGACTT GTGCTTTTTT GGACAGTGGA GGCACAAAAT  
5651 GTGAAATTCT TCAATTATTA AAGCCAATGT GTGAAGTGAA AACTGACTTT  
5701 TGTCAAGCTG TACTTCCATA CTTGATTCAT GATATTTTAC TCCAAGATAC  
5751 AAATGAATCA TGGAGAAATC TGCTTTCTAC ACATGTTTCAG GGATTTTTCA  
5801 CCAGCTGTCT TCGACACTTC TCGCAAACGA GCCGATCCAC AACCCTTGCA  
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5901 ATCACAAAGA ACAATGCTTG CTGTTGTGGA CTACATGAGA AGACAAAAGA  
5951 GACCTTCTTC AGGAACAATT TTTAATGATG CTTTCTGGCT GGATTTAAAT  
6001 TATCTAGAAG TTGCCAAGGT AGCTCAGTCT TGTGCTGCTC ACTTTACAGC  
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6151 AGCTTGAGTG AAAAAAGTAA AGAAGAACT GGAATAAGTT TACAGGATCT  
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6301 CACGAAGCAA TGTGGGGCAA AGCCCTAGTA ACATATGACC TCGAAACAGC  
6351 AATCCCTCA TCAACACGCC AGGCAGGAAT CATTGAGGCC TTGCAGAAAT  
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6451 AATAAAGACT GGTGTCCTGA ACTAGAAGAA CTTCAATTACC AAGCAGCATG  
6501 GAGGAATATG CAGTGGGACC ATTGCACTTC CGTCAGCAA GAAGTAGAAG  
6551 GAACCAAGTTA CCATGAATCA TTGTACAATG CTCTACAATC TCTAAGAGAC  
6601 AGAGAATTCT CTACATTTTA TGAAAGTCTC AAATATGCCA GAGTAAAAGA  
6651 AGTGGAAGAG ATGTGTAAGC GCAGCCTTGA GTCTGTGTAT TCGCTCTATC  
6701 CCACACTTAG CAGGTGTCAG GCCATTGGAG AGCTGGAAAG CATTGGGGAG  
6751 CTTTCTCAA GATCAGTCAC ACATAGACAA CTCTCTGAAG TATATATTAA  
6801 GTGGCAGAAA CACTCCCAGC TTCTCAAGGA CAGTGATTTT AGTTTTCAGG  
6851 AGCCTATCAT GGCTCTACGC ACAGTCATTT TGGAGATCCT GATGGAAAAG



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Figure 6b (v1)

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6901  GAAATGGACA  ACTCACAAAG  AGAATGTATT  AAGGACATTC  TCACCAAACA
6951  CCTTGTTAGAA  CTCTCTATAC  TGGCCAGAAC  TTTCAAGAAC  ACTCAGCTCC
7001  CTGAAAGGGC  AATATTTTCA  ATTAAACAGT  ACAATTTCAGT  TAGCTGTGGA
7051  GTCTCTGAGT  GGCAGCTGGA  AGAAGCACAA  GTATTCTGGG  CAAAAAAGGA
7101  GCAGAGTCTT  GCCCTGAGTA  TTCTCAAGCA  AATGATCAAG  AAGTTGGATG
7151  CCAGCTGTGC  AGCGAACAAT  CCCAGCCTAA  AACTTACATA  CACAGAATGT
7201  CTGAGGGTTT  GTGGCAACTG  GTTAGCAGAA  ACGTGCCTAG  AAAATCCTGC
7251  GGTCAATCATG  CAGACCTATC  TAGAAAAGGC  AGTAGAAGTT  GCTGGAAATT
7301  ATGATGGAGA  AAGTAGTGAT  GAGCTAAGAA  ATGGA AAAAT  GAAGGCATTT
7351  CTCTCAATTAG  CCCGGTTTTT  AGATACTCAA  TACCAAAGAA  TTGAAAAC TA
7401  CATGAAATCA  TCGGAATTTG  AAAACAAGCA  AGCTCTCCTG  AAAAGAGCCA
7451  AAGAGGAAGT  AGGTCTCCTT  AGGGAACATA  AAATTCAGAC  AAACAGATAC
7501  ACAGTAAAGG  TTCAGCGAGA  GCTGGAGTTG  GATGAATTAG  CCCTGCGTGC
7551  ACTGAAAGAG  GATCGTAAAC  GCTTCTTATG  TAAAGCAGTT  GAAAATTATA
7601  TCAACTGCTT  ATTAAGTGGA  GAAGAACATG  ATATGTGGGT  ATTCCGGCTT
7651  TGTTCCTCT  GGCTTGAAAA  TTCTGGAGTT  TCTGAAGTCA  ATGGCATGAT
7701  GAAGAGAGAC  GGAATGAAGA  TTCCAACATA  TAAATTTTTG  CCTCTTATGT
7751  ACCAATTGGC  TGCTAGAATG  GGGACCAAGA  TGATGGGAGG  CCTAGGATTT
7801  CATGAAGTCC  TCAATAATCT  AATCTCTAGA  ATTTCAATGG  ATCACCCCCA
7851  TCACACTTTG  TTTATTATAC  TGGCCTTAGC  AAATGCAAAC  AGAGATGAAT
7901  TTCTGACTAA  ACCAGAGGTA  GCCAGAAGAA  GCAGAATAAC  TAAAAATGTG
7951  CCTAAACAAA  GCTCTCAGCT  TGATGAGGAT  CGAACAGAGG  CTGCAAATAG
8001  AATAATATGT  ACTATCAGAA  GTAGGAGACC  TCAGATGGTC  AGAAGTGT TG
8051  AGGCACTTTG  TGATGCTTAT  ATTATATTAG  CAAACTTAGA  TGCCACTCAG
8101  TGGAAGACTC  AGAGAAAAGG  CATAAATATT  CCAGCAGACC  AGCCAATTAC
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8301  TGTAGGTTCC  GATGGCAAGG  AGAGGAGACA  GCTTGTTAAG  GGCCGTGATG
8351  ACCTGAGACA  AGATGCTGTC  ATGCAACAGG  TCTTCAGAT  GTGTAATACA
8401  TTA CTG CAGA  GAAACACGGA  AACTAGGAAG  AGGAAATTAA  CTATCTGTAC
8451  TTATAAGGTG  GTTCCCTCT  CTCAGCGAAG  TGGTGTCTT  GAATGGTGCA
8501  CAGGA ACTGT  CCCCAT TGGT  GAATTTCTTG  TTAACAATGA  AGATGGTGCT
8551  CATAAAAGAT  ACAGGCCAAA  TGATTTTCAGT  GCCTTTTCAGT  GCCAAAAGAA

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Figure 6b (vii)

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8701 AAATTCCTGG ATCCAGCTAT TTGGTTTGAG AAGCGATTGG CTTATACGCG  
8751 CAGTGTAGCT ACTTCTTCTA TTGTGGTTA CATACTTGA CTTGGTGATA  
8801 GACATGTACA GAATATCTTG ATAAATGAGC AGTCAGCAGA ACTTGACAT  
8851 ATAGATCTAG GTGTTGCTTT TGAACAGGGC AAAATCCTTC CTA CTCTCTGA  
8901 GACAGTTCCT TTTAGACTCA CCAGAGATAT TGTGGATGGC ATGGGCATTA  
8951 CGGGTGTGA AGGTGTCTTC AGAAGATGCT GTGAGAAAAC CATGGAAGTG  
9001 ATGAGAACT CTCAGGAAAC TCTGTAAACC ATTGTAGAGG TCCTTCTATA  
9051 TGATCCACTC TTTGACTGGA CCATGAATCC TTTGAAAGCT TTGTATTTAC  
9101 AGCAGAGGCC GGAAGATGAA ACTGAGCTTC ACCCTACTCT GAATGCAGAT  
9151 GACCAAGAAT GCAAACGAAA TCTCAGTGAT ATTGACCAGA GTTTCGACAA  
9201 AGTAGCTGAA CGTGTCTTAA TGAGACTACA AGAGAACTG AAAGGAGTGG  
9251 AAGAAGGCAC TGTGCTCAGT GTTGGTGGAC AGGTGAATTT GCTCATACAG  
9301 CAGGCCATAG ACCCCAAAAA TCTCAGCCGA CTTTCCAG GATGGAAAGC  
9351 TTGGGTGTGA TCTTCAGTAT ATGAATTACC CTTTC



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Figure 7a

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FT                                  KQSQHMTEVVRRCPPHERCSDSDGLAPPQHLIRVEGNLRVEYLLDRNTFRHSVVVPYEP
FT                                  PEVGSDCTTIHYNMCMSSCMGGMNRRPILTIITLEDSSGNLLGRNSFEVRVCACPGRD
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SQ   Sequence 1303 BP; 292 A; 403 C; 348 G; 260 T; 0 other;

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M14695 Length: 1303 July 10, 1998 12:29 Type: N Check: 4902 ..

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51 GGGAGCGTGC TTTCACGAC GGTGACACGC TTCCCTGGAT TGGCAGCCAG
101 ACTGCCCTTC GGGTCACTGC CATGGAGGAG CCGCAGTCAG ATCCTAGCGT
151 CGAGCCCCCT CTGAGTCAGG AAACATTTTC AGACCTATGG AAACACTTTC
201 CTGAAAACAA CGTTCTGTCC CCCTTGCCGT CCCAAGCAAT GGATGATTTG
251 ATGCTGTCCC CGGACGATAT TGAACAATGG TTCACTGAAG ACCCAGGTCC

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Figure 7b (1 )





Figure 7b (11)

301 AGATGAAGCT CCCAGAATGC CAGAGGCTGC TCCCCCGTG GCCCCTGCAC  
351 CAGCGACTCC TACACCGGCG GCCCCTGCAC CAGCCCCCTC CTGGCCCCCTG  
401 TCATCTTCTG TCCCTTCCCA GAAAACCTAC CAGGGCAGCT ACGGTTTCCG  
451 TCTGGGCTTC TTGCATTCTG GGACAGCCAA GTCTGTGACT TGCACGTA CT  
501 CCCCTGCCCT CAACAAGATG TTTTGCCAAC TGGCCAAGAC CTGCCCTGTG  
551 CAGCTGTGGG TTGATTCCAC ACCCCCGCCC GGCACCCGCG TCCGCGCCAT  
601 GGCCATCTAC AAGCAGTCAC AGCACATGAC GGAGGTGTG AGGCGCTGCC  
651 CCCACCATGA GCGCTGCTCA GATAGCGATG GTCTGGCCCC TCCTCAGCAT  
701 CTTATCCGAG TGGAAGGAAA TTTGCGTGTG GAGTATTTGG ATGACAGAAA  
751 CACTTTTTCGA CATAGTGTGG TGGTGCCCTA TGAGCCGCCT GAGGTTGGCT  
801 CTGACTGTAC CACCATCCAC TACAACTACA TGTGTAACAG TTCCTGCATG  
851 GGCGGCATGA ACCGGAGGCC CATCCTCACC ATCATCACAC TGGAAGACTC  
901 CAGTGGTAAT CTACTGGGAC GGAACAGCTT TGAGGTGCGT GTTTGTGCCT  
951 GTCCTGGGAG AGACCGGCGC ACAGAGGAAG AGAATCTCCG CAAGAAAGGG  
1001 GAGCCTCACC ACGAGCTGCC CCCAGGGAGC ACTAAGCGAG CACTGCCCAA  
1051 CAACACCAGC TCCTCTCCCC AGCCAAAGAA GAAACCACTG GATGGAGAAT  
1101 ATTTACACCT TCAGATCCGT GGGCGTGAGC GCTTCGAGAT GTTCCGAGAG  
1151 CTGAATGAGG CCTTGGA ACT CAAGGATGCC CAGGCTGGGA AGGAGCCAGG  
1201 GGGGAGCAGG GCTCACTCCA GCCACCTGAA GTCCAAAAAG GGTCACTCTA  
1251 CCTCCCGCCA TAAAAA ACTC ATGTTCAAGA CAGAAGGGCC TGACTCAGAC  
1301 TGA



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Figure 8a (i)

```

!!NA_SEQUENCE 1.0
ID   HS498441   standard; RNA; HUM; 8210 BP.
XX
AC   U49844;
XX
NI   g1235901
XX
DT   20-MAY-1996 (Rel. 47, Created)
DT   20-MAY-1996 (Rel. 47, Last updated, Version 1)
XX
→ DE   Human FRAP-related protein (FRP1) mRNA, complete cds.
XX
KW
XX
OS   Homo sapiens (human)
OC   Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Primates;
OC   Catarrhini; Hominidae; Homo.
XX
RN   [1]
RP   1-8210
RA   Cimprich K.A., Shin T.B., Keith C.T., Schreiber S.L.;
RT   "cDNA cloning and gene mapping of a candidate human cell cycle
RT   checkpoint protein";
RL   Proc. Natl. Acad. Sci. U.S.A. 93:2850-2855(1996).
XX
RN   [2]
RP   1-8210
RA   Cimprich K.A., Shin T.B., Keith C.T., Schreiber S.L.;
RT   ;
RL   Submitted (22-FEB-1996) to the EMBL/GenBank/DBJ databases.
RL   Karlene A. Cimprich, Chemistry, Harvard University, 12 Oxford Street,
RL   Cambridge, MA 02138, USA
XX
DR   SPTREMBL; Q13535; Q13535.
XX
FH   Key          Location/Qualifiers
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FT                   /chromosome="3"
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FT                   /map="3q22-q24"
FT   CDS              106. .8040
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FT                   /db_xref="SPTREMBL:Q13535"
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FT                   /gene="FRP1" or ATR
FT                   /product="FRAP-related protein"
FT                   /translation="MGEHGLELASMPALRELGSATPEEYNTIVVQKPRQILCQFIDRIL
FT                   TDVNVVAVELVKKTDQSPTSVMLLDFIQHIMKSSPLMFVNVVSGSHEAKGSCIEFSNWII
FT                   TRLLRIAATPSCHLLHKKICEVICSLFLFKSKSPAIFGVLTKEILLQFEDLVYLHRRN
FT                   VMGHAVEWPPVMSRFLSQLDEHMGYLSAPLQMSMQLNLEFIEVTLLMVLTRIATVFF
FT                   RRQELLWQIGCVLLEYGSPKIKSLAISFLTELFQGLPAQPASTFFSFLLELLKHLV
FT                   EMDTDQLKLYEPLSKLIKTLFPFEAEAYRNIEPVYLNMLEKLCVMFEDGVLMLRLKSD
FT                   LLKAAALCHLLQYFLKFVPAGYESALQVRKVYVRNICKALLDVLGIEVDAEYLLGPLYAA
FT                   LKMESEIIEEIQCQTQQENLSSNSDGISPKRRRLSSSLNPSKRAPKQTEEEKHVDNQ
FT                   KSLWSALKQKAESLQISLEYSGLNPNVIEMLGLI AVLQLTALCTVHCSHQNMNCRFT
FT                   KDCQHKSKKKPSVVITWMSLDFYTKVLKSCRSLLSVQKLDLEATIDKVKIYDALIYM
FT                   QVNSSFEDHILEDLGLSLPWIYSHSDDGCLKLTFAANLLTLSCRISDSYSPQAQSR
FT                   CVFLLTLFPRRIFLEWRTAVYNWALQSSHEVIRASCVSGFFILLQQQNSCNRPVKILID
FT                   KVKDDSDIVKKEFASILGQLVCTLHGMFYLTSSLTEPFSEGHVDFCRNLKATSOHEC
FT                   SSSQLKASVCKPFLFLKKKIPSPVKLAFIDNLHHLCKHLDFREDETQKAVLGTLNL
FT                   MEDPDKDVRVAFSGNIKHILESLDSEDFIKELFVLRMKEAYTHAQISRNNELKDTLIL
FT                   TTGDIGRAAKGDLVPFALLHLLHCLLSKSASVSGAAYTEIRALVAAKSVKLQSFSSQYK
FT                   KPICQFLVESLHSSQMTALPNTPCQADVRKQDVHQREMAINTLSEIANVDFPDLNR
FT                   FLTRTLQVLLPDLAAKASPAASALIRTLGKQLNVNRRREILINNFKYIFSHLVCSCKDE

```



Figure 8a (ii)

FT LERALHYLKNETEIELGSLLRQDFQGLHNEILLRIGEHYQQVFNGLSILASFSSDDPY  
 FT QGPRDIISPELMADYLOPKLLGILAFFNMQLLSSSVGIEDKKMALNSLSMKLMGPKH  
 FT VSSVRVKMMTTLRGTGLRFKDDFPELCCRAWDCFVRCLDHACLGSLLSHVIVALLPLIHI  
 FT QPKETAAlFHYLIIEENRDAVQDFLHEIYFLPDHPELKKIKAVLQEURKETSESTDLQTT  
 FT LQLSMKAIQHENVVRIHALTSLKETLYKNQEKLIKATDSETVEPIISQLVTVLLKGC  
 FT QDANSQARLLCGECLGELGAIDPGRLDFTTETQGGKDFTFVTGVEDSSFAYGLLMELTR  
 FT AYLAYADNSRAQDSAAAYAIQELLSIYDCREMETNGPGHQLWRRFPEHVREILEPHLNTR  
 FT YKSSQKSTDWSGVKKPIYLSKLGSNFAEWSASWAGYLI TKVRHDLASKIFTCCSIMMKH  
 FT DFKVTIYLLPHILVYVLLGCNQEDQQEVYAEIMAVLKHDDQHTINTQDIASDLQCLSTQ  
 FT TVFSMLDHLTQWARHKFQALKAKECPHSHKSNRNKVDMSVSTVDYEDYQSVTRFLDLIPQ  
 FT DTLAVASFRSKAYTRAVMHFESFITEKKQNIQEHGLFLQKLYAAMHEPDGVAGVSAIRK  
 FT AEPSLKEQILEHESLGLLRDATAICYDRAIQLEPDQIIHYHGVVKSMLGLGQLSTVITQV  
 FT NGVHANRSEWTDENLTYRVEAAWKLQWDLVENYLAADGKSTTWSVRLGQLLSAKKRD  
 FT ITAFYDSLKLVRAEQIVPLSAASFERSYQRGYEVIVRLHMLCELEHSIKPLFQHSFGD  
 FT SSQEDSLNWVARLEMTQNSYRAKEPILALRRALLSLNKRPDYNEMVGECWLQ SARVARK  
 FT AGHHQTAYNALLNAGESRLAELYVERAKWLWSKGDVHQALIVLQKGVELCFPENETPPE  
 FT GKNMLIHGRAMLLVGRFMEETANFESNAIMKKYKDVTA CLPEWEDGHFYLA KYDYKLMF  
 FT MVTDNKMEKQGD LIRYIVLHFGRSLO YGNQFIYQSMRMLTLWLDYGT KAYEWEKAGRS  
 FT DRVQMRNDLGKINKVITEHTNYLAPYQFLTA FSQ LISRICSHDEVFVVLMEIIAKVFL  
 FT AYPQQAMWMMTAVSKSSYPMRVNRCKEILNKAIHMKKSLEKFVG DATRLTDKLEL CNK  
 FT PVDGSSSTLSMSTHF KMLKKLVEEATFSEILIPQSVMIPTLPSILGTHANHASHEPFP  
 FT GHWAYIAGFDDMVEILASLQPKKISLKGSDGKFYIMCKPKDDLKDCRLMEFNSLIN  
 FT KCLRKDAESRRRELHIRTAYAVIPLNDECGIIEWVNNTAGLRPI LTKLYKEKGVYMTGKE  
 FT LRQCMLPKSAALSEKLKVFREFLLPRHPIFHEWFLRTFPDPTSWYSSRSAYCRSTAVM  
 FT SMVGYILGLGDRHGENILFDSLGTGECVHVDNFCLFNKGETFEVPEIVPFR LTHNMVNGM  
 FT GPMGTGELFRACEVTMLMRDQREPLMSVLKTF LHDPLVWSKPVKGHSKAPLNETGE  
 FT VVNEKAKTHVLDIEQRLQGVITKTRNRVTGLPLSIEGHVHYLIQEATDENLLCQMYLGWT  
 FT PYM"  
 XX  
 SQ Sequence 8210 BP; 2511 A; 1555 C; 1738 G; 2406 T; 0 other;

U49844 Length: 8210 July 10, 1998 12:08 Type: N Check: 4511 ..

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 51 TGGAGACGCC GGAACCCGCG GTTGGCGTGG TTGACTAGTG CCTCGCAGCC  
 101 TCAGCATGGG GGAACATGGC CTGGAGCTGG CTTCCATGAT CCCC GCCCTG  
 151 CGGGAGCTGG GCAGTGCCAC ACCAGAGGAA TATAATACAG TTGTACAGAA  
 201 GCCAAGACAA ATTCTGTGTC AATTCATTGA CCGGATACIT ACAGATGTAA  
 251 ATGTTGTTGC TGTAGAACTT GTAAAGAAAA CTGACTCTCA GCCAACCTCC  
 301 GTGATGTTGC TTGATTTTCAT CCAGCATATC ATGAAATCCT CCCCACTTAT  
 351 GTTTGTAAAT GTGAGTGGA GGCATGAGGC CAAAGGCAGT TGTATTGAAT  
 401 TCAGTAATTG GATCATAACG AGACTTCTGC GGATTGCAGC AACTCCCTCC  
 451 TGTCATTTGT TACACAAGAA AATCTGTGAA GTCATCTGTT CATTATTATT  
 501 TCTTTT TAAA AGCAAGAGTC CTGCTATTTT TGGGGTACTC ACAAAGAAT  
 551 TATTACAAC TTTTGAAGAC TTGGTTTACC TCCATAGAAG AAATGTGATG  
 601 GGTCATGCTG TGGAATGGCC AGTGGTCATG AGCCGATTTT TAAGTCAATT  
 651 AGATGAACAC ATGGGATATT TACAATCAGC TCCTTTGCAG TTGATGAGTA  
 701 TGCAAAATTT AGAATTTATT GAAGTCACIT TATTAATGGT TCTTACTCGT  
 751 ATTATTGCAA TTGTGTTTTT TAGAAGGCAA GAACTCTTAC TTTGGCAGAT  
 801 AGGTTGTGTT CTGCTAGAGT ATGGTAGTCC AAAAATTAAA TCCCTAGCAA

Figure 8b(1)



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Figure 8b (ii)

851 TTAGCTTTTT AACAGAACTT TTTCAGCTTG GAGGACTACC AGCACAACCA  
901 GCTAGCACTT TTTTCAGCTC ATTTTGGAA TTATTAAAAC ACCTTGTTAGA  
951 AATGGATACT GACCAATTGA AACTCTATGA AGAGCCATTA TCAAAGCTGA  
1001 TAAAGACACT ATTTCCCTTT GAAGCAGAAG CTTATAGAAA TATTGAACCT  
1051 GTCTATTTAA ATATGCTGCT GGAAAACTC TGTGTCATGT TTGAAGACGG  
1101 TGTGCTCATG CGGCTTAAGT CTGATTTGCT AAAAGCAGCT TTGTGCCATT  
1151 TACTGCAGTA TTTCCTTAAA TTTGTGCCAG CTGGGTATGA ATCTGCTTTA  
1201 CAAGTCAGGA AGGTCTATGT GAGAAATATT GTTAAAGCTC TTTTGGATGT  
1251 GCTTGAATT GAGGTAGATG CAGAGTACTT GTTGGGCCCA CTTTATGCAG  
1301 CTTTGAAAAT GGAAAGTATG GAAATCATTG AGGAGATTCA ATGCCAAACT  
1351 CAACAGGAAA ACCTCAGCAG TAATAGTGAT GGAATATCAC CCAAAGGCG  
1401 TCGTCTCAGC TCGTCTCTAA ACCCTTCTAA AAGAGCACCA AAACAGACTG  
1451 AGGAAATTAA ACATGTGGAC ATGAACCAAA AGAGCATATT ATGGAGTGCA  
1501 CTGAAACAGA AAGCTGAATC CCTTCAGATT TCCCTTGAAT ACAGTGGCCT  
1551 AAAGAATCCT GTTATTGAGA TGTTAGAAGG AATTGCTGTT GTCTTACAAC  
1601 TGACTGCTCT GTGTACTGTT CATTGTTCTC ATCAAAACAT GAACTGCCGT  
1651 ACTTTCAAGG ACTGTCAACA TAAATCCAAG AAGAAACCTT CTGTAGTGAT  
1701 AACTTGGATG TCATTGGATT TTTACACAAA AGTGCTTAAG AGCTGTAGAA  
1751 GTTGTGTTAGA ATCTGTTTCA AACTGGACC TGGAGGCAAC CATTGATAAG  
1801 GTGGTGAAAA TTTATGATGC TTTGATTTAT ATGCAAGTAA ACAGTTTATT  
1851 TGAAGATCAT ATCCTGGAAG ATTTATGTGG TATGCTCTCA CTTCCATGGA  
1901 TTTATTCCCA TTCTGATGAT GGCTGTTTAA AGTTGACCAC ATTTGCCGCT  
1951 AATCTTCTAA CATTAGCTG TAGGATTTCA GATAGCTATT CACCACAGGC  
2001 ACAATCACGA TGTGTGTTTC TTCTGACTCT GTTTCCAAGA AGAATATTCC  
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2201 ATTCTGACAT TGTCAAGAAA GAATTTGCTT CTATACTTGG TCAACTTGTC  
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2301 CTCTGAACAC GGACATGTGG ACCTCTTCTG TAGGAACCTG AAAGCCACTT  
2351 CTCAACATGA ATGTTTATCT TCTCAACTAA AAGCTTCTGT CTGCAAGCCA  
2401 TTCTTTTCC TACTGAAAAA AAAAATACCT AGTCCAGTAA AACTTGCTTT  
2451 CATAGATAAT CTACATCATC TTTGTAAGCA TCTTGATTTT AGAGAAGATG  
2501 AAACAGATGT AAAAGCAGTT CTGGAACCTT TATTAAATTT AATGGAAGAT





Figure 8b (111)

2551 CCAGACAAAG ATGTTAGAGT GGCTTTTAGT GGAAATATCA AGCACATATT  
2601 GGAATCCTTG GACTCTGAAG ATGGATTTAT AAAGGAGCTT TTTGTCTTAA  
2651 GAATGAAGGA AGCATATACA CATGCCCAA TATCAAGAAA TAATGAGCTG  
2701 AAGGATACCT TGATTCTTAC AACAGGGGAT ATTGGAAGGG CCGCAAAAGG  
2751 AGATTTGFTA CCATTTGCAC TCTTACACTT ATTGCATTGT TTGTTATCCA  
2801 AGTCAGCATC TGTCTCTGGA GCAGCATACA CAGAAATTAG AGCTCTGGTT  
2851 GCAGCTAAAA GTGTTAAACT GCAAAGTTTT TTCAGCCAGT ATAAGAAACC  
2901 CATCTGTCAG TTTTGGTAG AATCCCTTCA CTCTAGTCAG ATGACAGCAC  
2951 TTCCGAATAC TCCATGCCAG AATGCTGACG TGCGAAAACA AGATGTGGCT  
3001 CACCAGAGAG AAATGGCTTT AAATACGTTG TCTGAAATTG CCAACGTTTT  
3051 CGACTTTCCT GATCTTAATC GTTTTCTTAC TAGGACATTA CAAGTTCTAC  
3101 TACCTGATCT TGCTGCCAAA GCAAGCCCTG CAGCTTCTGC TCTCATTCGA  
3151 ACTTTAGGAA AACAATTAAA TGTCAATCGT AGAGAGATTT TAATAAACAA  
3201 CTTCAAATAT ATTTTTCTC ATTTGGTCTG TTCTTGTTCC AAAGATGAAT  
3251 TAGAACGTGC CCTTCATTAT CTGAAGAATG AAACAGAAAT TGAAC TGGGG  
3301 AGCCTGTTGA GACAAGATTT CCAAGGATTG CATAATGAAT TATTGCTGCG  
3351 TATTGGAGAA CACTATCAAC AGGTTTTTAA TGGTTTGTC A TACTTGCCT  
3401 CATTTGCATC CAGTGATGAT CCATATCAGG GCCCGAGAGA TATCATATCA  
3451 CCTGAACTGA TGGCTGATTA TTTACAACCC AAATTGTTGG GCATTTTGGC  
3501 TTTTTTTAAC ATGCAGTTAC TGAGCTCTAG TGTTGGCATT GAAGATAAGA  
3551 AAATGGCCTT GAACAGTTTG ATGTCTTTGA TGAAGTTAAT GGGACCCAAA  
3601 CATGTCAGTT CTGTGAGGGT GAAGATGATG ACCACACTGA GAACTGGCCT  
3651 TCGATTCAAG GATGATTTTC CTGAATTGTG TTGCAGAGCT TGGGACTGCT  
3701 TTGTTGCTG CCTGGATCAT GCTTGTCTGG GCTCCCTTCT CAGTCATGTA  
3751 ATAGTAGCTT TGTTACCTCT TATACACATC CAGCCTAAAG AAAGTGCAGC  
3801 TATCTTCCAC TACCTCATAA TTGAAAACAG GGATGCTGTG CAAGATTTTC  
3851 TTCATGAAAT ATATTTTTTA CCTGATCATC CAGAATTAAA AAAGATAAAA  
3901 GCCGTTCTCC AGGAATACAG AAAGGAGACC TCTGAGAGCA CTGATCTTCA  
3951 GACAACTCTT CAGCTCTCTA TGAAGGCCAT TCAACATGAA AATGTGATG  
4001 TTCGTATTCA TGCTCTTACA AGCTTGAAGG AAACCTTGTA TAAAAATCAG  
4051 GAAAACTGA TAAAGTATGC AACAGACAGT GAAACAGTAG AACCTATTAT  
4101 CTCACAGTTG GTGACAGTGC TTTTGAAAGG TTGCCAAGAT GCAAACTCTC  
4151 AAGCTCGGTT GCTCTGTGGG GAATGTTTAG GGAATTGGG GCGATAGAT



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Figure 8b (iv)

4201 CCAGGTCGAT TAGATTTCTC AACAACTGAA ACTCAAGGAA AAGATTTTAC  
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 4301 AGCTAACAAG AGCTTACCTT GCGTACGCTG ATAATAGCCG AGCTCAAGAT  
 4351 TCAGCTGCCT ATGCCATTCA GGAGTTGCTT TCTATTTATG ACTGTAGAGA  
 4401 GATGGAGACC AACGGCCCAG GTCACCAATT GTGGAGGAGA TTTCTTGAGC  
 4451 ATGTTCCGGA AATACTAGAA CCTCATCTAA ATACCAGATA CAAGAGTTCT  
 4501 CAGAAGTCAA CCGATTGGTC TGGAGTAAAG AAGCCAATTT ACTTAAGTAA  
 4551 ATTGGGTAGT AACTTTGCAG AATGGTCAGC ATCTTGGGCA GGTATCTTA  
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 4701 TCTGGTGTAT GTCTTACTGG GTTGTAATCA AGAAGATCAG CAGGAGGTTT  
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 5301 GATCATTCAT TATCATGGTG TAGTAAAGTC CATGTTAGGT CTTGGTCAGC  
 5351 TGTCTACTGT TATCACTCAG GTGAATGGAG TGCATGCTAA CAGGTCCGAG  
 5401 TGGACAGATG AATTAAACAC GTACAGAGTG GAAGCAGCTT GGAAATTGTC  
 5451 ACAGTGGGAT TTGGTGGAAA ACTATTTGGC AGCAGATGGA AAATCTACAA  
 5501 CATGGAGTGT CAGACTGGGA CAGCTATTAT TATCAGCCAA AAAAAGAGAT  
 5551 ATCACAGCTT TTTATGACTC ACTGAAACTA GTGAGAGCAG AACAAATTGT  
 5601 ACCTCTTTCA GCTGCAAGCT TTGAAAGAGG CTCTACCAA CGAGGATATG  
 5651 AATATATTGT GAGATTGCAC ATGTTATGTG AGTTGGAGCA TAGCATCAAA  
 5701 CCACTTTTCC AGCATTCTCC AGGTGACAGT TCTCAAGAAG ATTCTCTAAA  
 5751 CTGGGTAGCT OGA CTAGAAA TGACCCAGAA TTCTACAGA GCCAAGGAGC  
 5801 CTATCTGGC TCTCCGGAGG GCTTTACTAA GCTCAACAA AAGACCAGAT  
 5851 TACAATGAAA TGGTTGGAGA ATGCTGGCTG CAGAGTGOCA GGGTAGCTAG



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Figure 8b (v)

5901 AAAGGCTGGT CACCACCAGA CAGCCTACAA TGCTCTCCTT AATGCAGGGG  
5951 AATCACGACT CGCTGAACTG TACGTGGAAA GGGCAAAGTG GCTCTGGTCC  
6001 AAGGGTGATG TTCACCAGGC ACTAATTGTT CTTCAAAAAG GTGTTGAATT  
6051 ATGTTTTTCCT GAAAATGAAA CCCCACCTGA GGGTAAGAAC ATGTTAATCC  
6101 ATGGTCGAGC TATGCTACTA GTGGGCCGAT TTATGGAAGA AACAGCTAAC  
6151 TTTGAAAGCA ATGCAATTAT GAAAAAATAT AAGGATGTGA CCGCGTGCCT  
6201 GCCAGAATGG GAGGATGGGC ATTTTACCT TGCCAAGTAC TATGACAAAT  
6251 TGATGCCCCAT GGTCAAGAC AACAAAATGG AAAAGCAAGG TGATCTCATC  
6301 CGGTATATAG TTCTTCATTT TGGCAGATCT CTACAATATG GAAATCAGTT  
6351 CATATATCAG TCAATGCCAC GAATGTTAAC TCTATGGCTT GATTATGGTA  
6401 CAAAGGCATA TGAATGGGAA AAAGCTGGCC GCTCCGATCG TGTACAAATG  
6451 AGGAATGATT TGGGTAAAT AAACAAGGTT ATCACAGAGC ATACAAACTA  
6501 TTTAGCTCCA TATCAATTTT TGACTGCTTT TTCACAATTG ATCTCTCGAA  
6551 TTTGTCATTC TCACGATGAA GTTTTGTGTG TCTTGATGGA AATAATAGCC  
6601 AAAGTATTTT TAGCCTATCC TCAACAAGCA ATGTGGATGA TGACAGCTGT  
6651 GTCAAAGTCA TCTTATCCCA TGGGTGTGAA CAGATGCAAG GAAATCCTCA  
6701 ATAAAGCTAT TCATATGAAA AAATCCTTAG AGAAGTTTGT TGGAGATGCA  
6751 ACTCGCCTAA CAGATAAGCT TCTAGAATTG TGCAATAAAC CGGTTGATGG  
6801 AAGTAGTTCC ACATTAAGCA TGAGCACTCA TTTTAAATG CTTAAAAGC  
6851 TGGTAGAAGA AGCAACATTT AGTGAAATCC TCATTCTCTT ACAATCAGTC  
6901 ATGATACCTA CACTTCCATC AATTCTGGGT ACCCATGCTA ACCATGCTAG  
6951 CCATGAACCA TTTCTGGAC ATTGGGCCTA TATTGCAGGG TTGATGATA  
7001 TGGTGGAAT TCTTGCTTCT CTTCAGAAAC CAAAGAAGAT TTCTTTAAAA  
7051 GGCTCAGATG GAAAGTTCTA CATCATGATG TGTAAGCCAA AAGATGACCT  
7101 GAGAAAGGAT TGTAGACTAA TGGAATTCAA TTCCTTGATT AATAAGTGCT  
7151 TAAGAAAAGA TGCAGAGTCT CGTAGAAGAG AACTTCATAT TCGAACATAT  
7201 GCAGTTATTC CACTAAATGA TGAATGTGGG ATTATTGAAT GGGTGAACAA  
7251 CACTGCTGGT TTGAGACCTA TTCTGACCAA ACTATATAAA GAAAAGGGAG  
7301 TGTATATGAC AGGAAAAGAA CTTGCCAGT GTATGCTACC AAAGTCAGCA  
7351 GCTTTATCTG AAAAATCAA AGTATTCGA GAATTTCTCC TGCCAGGCA  
7401 TCCTCCTATT TTTCATGAGT GGTTCCTGAG AACATTCCCT GATCCTACAT  
7451 CATGGTACAG TAGTAGATCA GCTTACTGCC GTTCCACTGC AGTAATGTCA  
7501 ATGGTTGGTT ATATTCTGGG GCTTGGAGAC CGTCATGGTG AAAATATTCT



Figure 8b (v1)

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7551 CTTTGATTCT TTGACTGGTG AATGCGTACA TGTAGATTTT AATTGTCCTT
7601 TCAATAAGGG AGAAACCTTT GAAGTTCCAG AAATTGTGCC ATTTTCGCCTG
7651 ACTCATAATA TGGTTAATGG AATGGGTCCT ATGGGAACAG AGGGTCTTTT
7701 TCGAAGAGCA TGTGAAGTTA CAATGAGGCT GATGCGTGAT CAGCGAGAGC
7751 CTTTAATGAG TGTCTTAAAG ACTTTTCTAC ATGATCCTCT TGTGGAATGG
7801 AGTAAACCAG TGAAAGGGCA TTCCAAAGCG CCACTGAATG AAACCTGGAGA
7851 AGTTGTCAAT GAAAAGGCCA AGACCCATGT TCTTGACATT GAGCAGCGAC
7901 TACAAGGTGT AATCAAGACT CGAAATAGAG TGACAGGACT GCCGTTATCT
7951 ATTGAAGGAC ATGTGCATTA CCTTATACAG GAAGCTACTG ATGAAAACCT
8001 ACTATGCCAG ATGTATCTTG GTTGGACTCC ATATATGTGA AATGAAATTA
8051 TGTAAAAGAA TATGTTAATA ATCTAAAAGT AATGCATTTG GTATGAATCT
8101 GTGGTTGTAT CTGTTCAATT CTAAAGTACA ACATAAATTT ACGTTCTCAG
8151 CAACTGTTAT TTCTCTCTGA TCATTAATTA TATGTAAAAT AATATACATT
8201 CACTCGTGCC
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Figure 9a (1)

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!!NA_SEQUENCE 1.0
ID   HS349941   standard; RNA; HUM; 12780 BP.
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AC   U34994;
XX
NI   g995940
XX
DT   26-SEP-1995 (Rel. 45, Created)
DT   22-FEB-1997 (Rel. 51, Last updated, Version 4)
XX
DE   Human DNA-dependent protein kinase catalytic subunit (DNA-PKcs)
DE   mRNA, complete cds.
XX
KW
XX
OS   Homo sapiens (human)
OC   Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Primates;
OC   Catarrhini; Hominidae; Homo.
XX
RN   [1]
RP   1-12780
RX   MEDLINE; 95401275.
RA   Hartley K.O., Gell D., Smith G.C., Zhang H., Divecha N., Connelly M.A.,
RA   Admon A., Lees-Miller S.P., Anderson C.W., Jackson S.P.;
→ RT   "DNA-dependent protein kinase catalytic subunit: a relative of
RT   phosphatidylinositol 3-kinase and the ataxia telangiectasia gene
RT   product";
RL   Cell 82:849-856(1995).
XX
RN   [2]
RP   1-12780
RA   Gell D.;
RT   ;
RL   Submitted (29-AUG-1995) to the EMBL/GenBank/DDBJ databases.
RL   Dave Gell, Zoology, Wellcome/CRC, Tennis Court Road, Cambridge CB2 1QR,
RL   UK
XX
DR   SPTREMBL; Q13327; Q13327.
XX
CC   NCBI gi: 995940
XX
FH   Key          Location/Qualifiers
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FT   source          1. .12780
FT                   /organism="Homo sapiens"
FT                   /chromosome="8"
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FT                   /map="8q11"
FT   CDS              53. .12343
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FT                   /db_xref="PID:g995941"
FT                   /db_xref="SPTREMBL:Q13327"
FT                   /note="DNA-activated protein kinase catalytic subunit; PI
FT                   kinase family member; partial genomic sequence located in
FT                   GenBank Accession Number L27425; Method: conceptual
FT                   translation supplied by author. NCBI gi: 995941"
FT                   /gene="DNA-PKcs"
FT                   /product="DNA dependent protein kinase catalytic subunit"
FT                   /translation="MAGSGAGVRCSSLRLQETLSAADRCGAALAGHQIRGLGQECVLS
FT                   SSPAVLALQTSLVFSRDFGLLVFVRKSLNSIEFRECREEILKFLCIFLEKMGQKIAPYS
FT                   VEIKNTCTSVYTKDRAAKCKIPALDLLIKLLQTFRSSLMDDEFKIGELFSKFYGEALAK
FT                   KKIPDTVLEKVVYELLGLLGEVHPSEMINNAENLFRAFLGELKTQMTSAVREPKLPLVLAG
FT                   CLKGLSSLLCNFTKSMEEDPQTSREIFNFVLKAIRPQIDLKRYAVPSAGLRLFALHASQ
FT                   FSTCLLDNYVSLFEVLLKWCANTNVELKKAALSALESFLKQVSNMVAKNAMHKNKLOY
FT                   FMEQFYGIIRNVDSNNKELSIATIRGYGLFAGPCKVINAKDQDFMYVELIQRCQKQFLTQ
FT                   TDTGDIRVYQMPISFLQSVASVLLYLDTVPEVYTPVLEHLVVMQIDSFQYSPKMQLVCC
FT                   RAIVKVFLALAAKGPVLRNCISTVVHQLIRICSKPVVLPKGPESSESDHRASGEVRTG

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Figure 9 (ii)

FT KWKVPTYKYDYLDFRHLSSDQMMDSILADEAFFSVNSSSESINHLLYDEFVKSVLKIV  
 FT EKLDLTLEIQTVGEQENGDEAPGVWMIPTSDPAANLHPAKPKDFSAFINLVEFCREILP  
 FT EKQAEFFEPWVYSFSYELILQSTRPLISGFYKLLSITVRNAKKIKYFEGVSPKSLKHS  
 FT PEDPEKYSCFALFVKGKEVAVKMKQYKDELLASCLTFLLSLPHNIIELDVRAYVPALQ  
 FT MAFKLGLSYTPLAEVGLNALAEWSIYIDRHVMQPYKIDILPCLDGYLKTSALSDETKNN  
 FT WEVSALSRAAQKGFNKVVLKHLKKTKNLSSNEAISLEEIRVVMQGLSGGQINKNLL  
 FT TVTSSDEMMSYVAWDREKRLSFAVPPFREMKEPVIPLDVFLPRVTEALATASDRQTKVAA  
 FT CELLHSMVMFMLGKATQMPGGQGAPMYQLYKRTFPVLLRLACDQVQVTRQLYEPLVM  
 FT QLIHWFNTNNKFFESQDVTSLLEAILDGIIVDPVDSLDRDFCGRCIREFLKWSIKQITPOQ  
 FT QEKSPVNTKSLFKRLYSLALHPNAFKRLGASLAFNNIYREFREEESLVEQVFVEALVY  
 FT MESLALAHADEKSLGTIQCCDAIDHLCRIIEKKHVSLNKAHKRRLPRGFPPSASLCLL  
 FT DLVKWLLAHCGRPQTECRHKSIELFYKFVPLLPGNRSPNLWLKVDLKEEGVSFLINTFE  
 FT GGGCGQPSGILAQPTLLYLGRPFSLQATLCWLDLLLAALAEYCYNFTIGERTVGLAQVLT  
 FT EAQSSLLKAVAFLESIAMHDIIEAEKCFGTGAAGNRTSPQEGERYNYSKCTVVVRIME  
 FT FTTTLLNTSPGFWKLLKDLNTHLMRVLVQTLCEPASIGFNIQDVQVMAHLPDVQVNL  
 FT MKALKMSPYKDILETHLREKITAQSIEELCAVNLYGPDQVDRSLAAVVSACKQLHRA  
 FT GLHNLPSQSTDLHHSVGTTELLSLVYKGIAPGDERQCLPSLDLSCQQLASGLLELAF  
 FT FGGLCERLVSLLNPAVLSTASLGSSQGSVIHFSHGEYFYSLFSETINTELLKNLDLAV  
 FT LELMQSSVDNTKMSAVLNGMLDQSFREANQKHQGLKLATTILOHKKCDSWWAKDSP  
 FT LETKMAVLALLAKILOIDSSVSFNTSHGSFPEVFTTYISLLADTKLDLHLKGQAVTLLP  
 FT FFTSLTGGSLLEELRRVLEQLIVAHFPMQSRFPPTPRFNNVDCMKFLDALELSQSP  
 FT RQSFVDRSLLTLRQWCSLDALREFFSTIVVDAIDVLKSRFTKLNESTFDTQITKMGYY  
 FT KILDVMSRLPKDDVHAKESKINQVHFGSCITEGNETKTLIKLCYDAFTENMAGENQL  
 FT LERRRLYHCAAYNCAISVICCVFNEKIFYQGFLFSEKPEKNLLIFENLIDLKRRYNFPV  
 FT EVEVPMERKKKYIEIRKEAREAAANGSDGSPSYMSLSYLADSTLSEEMSQDFSTGVQS  
 FT YSYSSQDPRPATGRFRREQRDPVTHDDVLELEMDENRHECMAPLTALVKHMRSLGP  
 FT PQGEEDSVPRDLPWMKFLHGKLGNIPLNIRLFLAKLVINTEEVPRPYAKHWSPLL  
 FT QLAASENNGGEGIHVMVEIVATILSWTGLATPTGVPKDEVLANRLLNFMKHVFHPKR  
 FT AVFRHNLEIKITLVECKWDCLSIPYRLIFEKFSKDPNSKDNVSGIQLLGIVMANDLPP  
 FT YDPQCGIQSSEYFVALVNMSFVRYKEVYAAAAEVLGLILRYVMERKNILEESLCELVA  
 FT KQLKQHQNIMEDKFIVCLNKVTKSFPLADRFMNAVFFLLPKFHLKLCLEVVLCRV  
 FT EGMTLEYFQLKSKDFVQVMRHRDERQKVCLDIIYKMPKLPVELRELLNPVVEFVSHP  
 FT STTCREQMYNIMWIHDNYRDPESSETDNDSQEIFKLAKDVLIQGLIDENPGLQLIIRNF  
 FT WSHETRLPSNTDLRLALNSLYSPKIEVHFLSLATNLFLEMTSMSPDYNPMFEHPLSE  
 FT CEFQEYTTIDSDWRFRSTVLTPMFVETQASQGTLOTRTQEGSLSARWVPVAGQIRATQOQ  
 FT DFTLTQTADGRSSFDWLTGSSDPLVDHTSPSSDLSLFAHKRSERLQRAPLKSVGPDFG  
 FT KKRGLPGDEVDNKVGAAGRTDLRLRRRFRMRDQEKLSLMYARKGVAQKREKEIKSE  
 FT LKMKQDAQVVLRSYRHGDLPIQIKHSSLITPLQAVAQDPPIAKQLFSSLSFGILKE  
 FT MDKFKTLSEKNNTQKLLQDFNRLNITTSFFPPFVSCIQDISQCHVALLSLPAVLSA  
 FT GCLASLQOPVGIRLLEEALRLPLAELPAKRVRGKARLPDVLRLWELAKLYRSIGEYD  
 FT VLRGIFTSEIGTKQITQSALLAEARSYSEAAKQYDEALNKQDWDGEPTEAEKDFWEL  
 FT ASLDCYNHLAEWCSLEYCSTASIDSENPPDLNKIWESEPFYQETLYPYMIRSKLKLLOQ  
 FT EADQSLTLTIDKAMHGELOKALILELHYSQELSLLYLLQDDVDRAKYTYIQNGISFMQNY  
 FT SSIDVLLHQSRITKLQSVQALTEIQEFISFISKQGNLSSQVPLKRLNLTWNRYPDAKM  
 FT DPMNIWDDIITNRCFFLSKIEEKLTPLPEDNSMNVDQDGPDRMEVQEQEEDISSLIR  
 FT SCKFSMKMKMIDSARKQNNFSLAMKLLKELHKEKSTRDDWLVSQVQSYCRLSHCRSRQ  
 FT GCSEQVLTVLKTVSLDENNVSSYLXKNILAFRDQNTILLGTYRIANALSSSEPAELAE  
 FT IEEKARRILELSGSSSESEKVIAGLYQRAFQHLSEAVQAAEEEAQPPSWSCGPAAGV  
 FT IDAYMTLADFCQQLRKEEENASVTDSEALQAYPALVVEKMLKALKINSNEARLKFPRL  
 FT LQIIERYPEETLSLMTKEISSVPCWQFISWISHMVALLDKQAVAVQHSVEEITDNPQ  
 FT AIVYFFIISSESYFKDTSIGHKNKEFVARIKSKLDQGGVIQDFINALDQLSNPPELLFK  
 FT DWSNDVRAELAKTPVNKKNIEMMYERMYAALGDPKAPGLGAFRRKFIQTFGKEFDKHF  
 FT KGGSKLLRMKLSDFNDITNMLLLKMNKDSKPPGNLKECSPWMSDFKVEFLRNELEIPGQ  
 FT YDGRGKPLPEYHVRLAGFDERVTVMASLRRPKRIIRGHDEREHPFLVKGEDLRQDQ  
 FT VEQLFQVMNGILAQDSACSQRALQRTYSVVPMTSSDPRAPPEYKDWLTKMSGKHVVG  
 FT AYMLMYKGANRTTETVTSFRKRESKVPADLLKRAFVRMSTSPFAFLALRSHFASSHALIC  
 FT ISHWILGIGDRHLNFMVAMETGGVIGIDFGHAFGSATQFLPVPPELMPFRLTRQFINLM  
 FT LPMKETGLMYSIMVHALRAFRSDPGLLTNTMDVVFKEPSFDWKNFEQKMLKKGGSWIQE  
 FT INVAEKWYPRQKICYAKRKLAGANPAVITCDELILLGHEKAPAFRDYVAVARGSKDHNI  
 FT RAQEPESGLSEETQVKCLMDQATDPNILGRTWEGWEPWM\*  
 XX  
 SQ Sequence 12780 BP; 3612 A; 2769 C; 3084 G; 3314 T; 1 other;

U34994 Length: 12780 July 10, 1998 12:15 Type: N Check: 8189 .. Figure 9b(1)

1 ATTTCCGGGT CCGGCCCGAG CGGGCGCACG CGCGGGAGCG GGACTCGGCG



Figure 9b (ii)

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51  GCATGGCGGG CTCCGAGGCC GGTGTGCGTT GCTCCCTGCT GCGGCTGCAG
101 GAGACCTTGT CCGCTGCGGA CCGCTGCGGT GCTGCCCTGG CCGGTCATCA
151 ACTGATCCGC GGCTGGGGC AGGAATGCGT CCTGAGCAGC AGCCCCGCGG
201 TGCTGGCATT ACAGACATCT TTAGTTTTTT CCAGAGATTT CGGTTTGCTT
251 GTATTTGTCC GGAAGTCACT CAACAGTATT GAATTTCTGT AATGTAGAGA
301 AGAAATCCTA AAGTTTTTAT GTATTTTCTT AGAAAAATG GGCCAGAAGA
351 TCGCACCTTA CTCTGTTGAA ATTAAGAACA CTTGTACCAG TGTATTATACA
401 AAAGATAGAG CTGCTAAATG TAAAATCCA GCCCTGGACC TTCTTATTAA
451 GTTACTTCAG ACTTTTAGAA GTTCTAGACT CATGGATGAA TTTAAAATTG
501 GAGAATTATT TAGTAAATTC TATGGAGAAC TTGCATTGAA AAAAAAATA
551 CCAGATACAG TTTTAGAAAA AGTATATGAG CTCCTAGGAT TATTGGGTGA
601 AGTTCATCCT AGTGAGATGA TAAATAATGC AGAAAACCTG TTCCGCGCTT
651 TTCTGGGTGA ACTTAAGACC CAGATGACAT CAGCAGTAAG AGAGCCCAA
701 CTACCTGTTT TGGCAGGATG TCTGAAGGGG TTGTCCTCAC TTCTGTGCAA
751 CTTCACTAAG TCCATGGAAG AAGATCCCCA GACTTCAAGG GAGATTTTTA
801 ATTTTGTACT AAAGCAATT CGTCCTCAGA TTGATCTGAA GAGATATGCT
851 GTGCCCTCAG CTGGCTTGCG CCTATTTGCC CTGCATGCAT CTCAGTTTAG
901 CACCTGCCTT CTGGACAAC ACGTGTCTCT ATTTGAAGTC TTGTTAAAGT
951 GGTGTGCCCA CACAAATGTA GAATTGAAAA AAGCTGCACT TTCAGCCCTG
1001 GAATCCTTTC TGAAACAGGT TTCTAATATG GTGGCGAAAA ATGCAGAAAT
1051 GCATAAAAT AACTGCAGT ACTTTATGGA GCAGTTTTAT GGAATCATCA
1101 GAAATGTGGA TTGGAACAAC AAGGAGTTAT CTATTGCTAT CCGTGGATAT
1151 GGACTTTTTG CAGGACCGTG CAAGGTTATA AACGAAAAG ATGTTGACTT
1201 CATGTACGTT GAGCTCATTC AGCGCTGCAA GCAGATGTTT CTCACCCAGA
1251 CAGACACTGG TGACTIONGT GTTTATCAGA TGCCAAGCTT CCTCCAGTCT
1301 GTTGCAAGCG TCTTGCTGTA CCTTGACACA GTTCCTGAGG TGTATACTCC
1351 AGTTCTGGAG CACCTCGTGG TGATGCAGAT AGACAGTTTC CCACAGTACA
1401 GTCCAAAAAT GCAGCTGGTG TGTTCAGAG CCATAGTGAA GGTGTTCTTA
1451 GCTTTGGCAG CAAAAGGGCC AGTTCTCAGG AATTGCATTA GTAGTGGT
1501 GCATCAGGGT TTAATCAGAA TATGTTCTAA ACCAGTGGTC CTTCCAAAGG
1551 GCCCTGAGTC TGAATCTGAA GACCACCGTG CTTCAGGGGA AGTCAGAACT
1601 GGCAATGGA AGGTGCCAC ATACAAAGAC TACGTGGATC TCTTCAGACA
1651 TCTCCTGAGC TCTGACCAGA TGATGGATTC TATTTTAGCA GATGAAGCAT

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Figure 9b (111)

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1701 TTTTCTCTGT GAATTCCTCC AGTGAAAGTC TGAATCATTT ACTTTATGAT  
1751 GAATTTGTAA AATCCGTTTT GAAGATTGTT GAGAAATTGG ATCTTACACT  
1801 TGAAATACAG ACTGTTGGGG AACAAGAGAA TGGAGATGAG GCGCCTGGTG  
1851 TTTGGATGAT CCCAACTTCA GATCCAGCGG CTAAC TTGCA TCCAGCTAAA  
1901 CCTAAAGATT TTTCCGCTTT CATTAACCTG GTGGAATTTT GCAGAGAGAT  
1951 TCTCCCTGAG AAACAAGCAG AATTTTTTGA ACCATGGGTG TACTCATTTT  
2001 CATATGAATT AATTTTGCAA TCTACAAGGT TGCCCTCAT CAGTGGTTTC  
2051 TACAAATTGC TTTCTATTAC AGTAAGAAAT GCCAAGAAAA TAAAATATTT  
2101 CGAGGGAGTT AGTCCAAAGA GTCTGAAACA CTCTCCTGAA GACCCAGAAA  
2151 AGTATTCTTG CTTTGCTTTA TTTGTGAAAT TTGGCAAAGA GGTGGCAGTT  
2201 AAAATGAAGC AGTACAAAGA TGAAC TTTTG GCCTCTTGTT TGACCTTTCT  
2251 TCTGTCTTG CCACACAACA TCATTGAACT CGATGTTAGA GCCTACGTTT  
2301 CTGCACTGCA GATGGCTTTC AAACTGGGCC TGAGCTATAC CCCCTTGCA  
2351 GAAGTAGGCC TGAATGCTCT AGAAGAATGG TCAATTTATA TTGACAGACA  
2401 TGTAATGCAG CCTTATTACA AAGACATTCT CCCCTGCCTG GATGGATACC  
2451 TGAAGACTTC AGCCTTGTC GATGAGACCA AGAATAACTG GGAAGTGTC  
2501 GCTCTTTCTC GGGCTGCCA GAAAGGATT AATAAAGTGG TGTAAAAGCA  
2551 TCTGAAGAAG ACAAGAACC TTTTCATCAA CGAAGCAATA TCCTTAGAAG  
2601 AAATAAGAAT TAGAGTAGTA CAAATGCTTG GATCTCTAGG AGGACAAATA  
2651 AACAAAAATC TTCTGACAGT CACGCTCTCA GATGAGATGA TGAAGAGCTA  
2701 TGTGGCCTGG GACAGAGAGA AGCGGCTGAG CTTTGCACTG CCCTTTAGAG  
2751 AGATGAAACC TGTCA TTTTCTGGATGT TCCTGCCTCG AGTCACAGAA  
2801 TTAGCGCTCA CAGCCAGTGA CAGACAACT AAAGTTGCAG CCTGTGAACT  
2851 TTTACATAGC ATGGTTATGT TTATGTTGGG CAAAGCCACG CAGATGCCAG  
2901 AAGGGGGACA GGGAGCCCCA CCCATGTACC AGCTCTATAA GCGGACGTTT  
2951 CCTGTGCTGC TTCGACTTGC GTGTGATGTT GATCAGGTGA CAAGGCAACT  
3001 GTATGAGCCA CTAGTTATGC AGCTGATICA CTGGTTCACT AACACAAGA  
3051 AATTTGAAAG TCAGGATACT GTTTCCTTAC TAGAAGCTAT ATTGGATGGA  
3101 ATTGTGGACC CTGTTGACAG TACTTTAAGA GATTTTGTG GTCGGTGTAT  
3151 TCGAGAATTC CTTAAATGGT CCATTAAGCA AATAACACCA CAGCAGCAGG  
3201 AGAAGAGTCC AGTAAACACC AAATCGCTTT TCAAGCGACT TTATAGCCTT  
3251 GCGCTTCACC CCAATGCTTT CAAGAGGCTG GGAGCATCAC TTGCCTTTAA  
3301 TAATATCTAC AGGGAATTCA GGAAGAAGA GTCTCTGGTG GAACAGTTTG  
3351 TGTTTGAAGC CTTGGTGATA TACATGGAGA GTCTGGCCTT AGCACATGCA





Figure 9b (iv)

3401 GATGAGAAGT CCTTAGGTAC AATTCAACAG TGTGTGTGATG CCATTGATCA  
3451 CCTATGCCGC ATCATGTGAAA AGAAGCATGT TTCTTTTAAAT AAAGCAAAGA  
3501 AACGACGTTT GCCGCGAGGA TTTCCACCTT CCGCATCATT GTGTTTATTG  
3551 GATCTGGTCA AGTGGCTTTT AGCTCATTGT GGGAGGCCCC AGACAGAATG  
3601 TCGACACAAA TCCATTGAAC TCTTTTATAA ATTCTGTTCCCT TTATTGCCAG  
3651 GCAACAGATC CCCTAATTTG TGGCTGAAAG ATGTTCTCAA GGAAGAAGGT  
3701 GTCTCTTTTC TCATCAACAC CTTTGAGGGG GGTGGCTGTG GCCAGCCCTC  
3751 GGGCATCCTG GCCCAGCCCA CCCTCTTGTA CCTTCGGGGG CCATTTCAGCC  
3801 TGCAGGCCAC GCTATGCTGG CTGGACCTGC TCCTGGCCGC GTTGGAGTGC  
3851 TACAACACGT TCATTGGCGA GAGAACTGTA GGAGCGCTCC AGGTCCTAGG  
3901 TACTGAAGCC CAGTCTTCAC TTTTGAAAGC AGTGGCTTTC TTCTTAGAAA  
3951 GCATTGCCAT GCATGACATT ATAGCAGCAG AAAAGTGCTT TGGCACTGGG  
4001 GCAGCAGGTA ACAGAACAAG CCCACAAGAG GGAGAAAGGT ACAACTACAG  
4051 CAAATGCACC GTTGTGGTCC GGATTATGGA GTTTACCACG ACTCTGCTAA  
4101 ACACCTCCCC GGAAGGATGG AAGCTCCTGA AGAAGGACTT GTGTAATACA  
4151 CACCTGATGA GAGTCCTGGT GCAGACGCTG TGTGAGCCCG CAAGCATAGG  
4201 TTTCAACATC GGAGACGTCC AGGTTATGGC TCATCTTCCT GATGTTTGTG  
4251 TGAATCTGAT GAAAGCTCTA AAGATGTCCC CATACAAAGA TATCCTAGAG  
4301 ACCCATCTGA GAGAGAAAAT AACAGCACAG AGCATTGAGG AGCTTTGTGC  
4351 CGTCAACTTG TATGGCCCTG ACGCGCAAGT GGACAGGAGC AGGCTGGCTG  
4401 CTGTTGTGTC TGCCTGTAAA CAGCTTCACA GAGCTGGGCT TCTGCATAAT  
4451 ATATTACCGT CTCAGTCCAC AGATTTGCAT CATTCTGTTG GCACAGAACT  
4501 TCTTTCCCTG GTTTATAAAG GCATTGCCCC TGGAGATGAG AGACAGTGTG  
4551 TGCCTTCTCT AGACCTCAGT TGTAAGCAGC TGGCCAGCGG ACTTCTGGAG  
4601 TTAGCCTTTG CTTTTGGAGG ACTGTGTGAG CGCCTTGTGA GTCTTCTCCT  
4651 GAACCCAGCG GTGCTGTCCA CGGCGTCCTT GGGCAGCTCA CAGGGCAGCG  
4701 TCATCCACTT CTCCCATGGG GAGTATTTCT ATAGCTTGTT CTCAGAAACG  
4751 ATCAACACGG AATTATTGAA AAATCTGGAT CTTGCTGTAT TGGAGCTCAT  
4801 GCAGTCTTCA GTGGATAATA CCAAAATGGT GAGTGCCGTT TTGAACGGCA  
4851 TGTTAGACCA GAGCTTCAGG GAGCGAGCAA ACCAGAAACA CCAAGGACTG  
4901 AAAGTTGCGA CTACAATTCT GCAACACTGG AAGAAGTGTG ATTCATGGTG  
4951 GGCCAAAGAT TCCCCTCTCG AAAGTAAAAT GGCAGTGCTG GCCTTACTGG  
5001 CAAAAATTTT ACAGATTGAT TCATCTGTAT CTTTAAATAC AAGTCATGGT



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Figure 9b (v)

5051 TCATTCCCTG AAGTCTTTAC AACATATATT AGTCTACTTG CTGACACAAA  
 5101 GCTGGATCTA CATTTAAAGG GCCAAGCTGT CACTCTTCTT CCATTCTTCA  
 5151 CCAGCCTCAC TGGAGGCAGT CTGGAGGAAC TTAGACGTGT TCTGGAGCAG  
 5201 CTCATCGTTG CTCACTTCCC CATGCAGTCC AGGGAATTTT CTCCAGGAAC  
 5251 TCCGCGGTTT AATAATTATG TGGACTGCAT GAAAAAGTTT CTAGATGCAT  
 5301 TGGAATTATC TCAAAGCCCT ATGTTGTTGG AATTGATGAC AGAAGTTCTT  
 5351 TGTCCGGAAC AGCAGCATGT CATGGAAGAA TTATTTCAAT CCAGTTTCAG  
 5401 GAGGATTGCC AGAAGGGGTT CATGTGTCAC ACAAGTAGGC CTTCGGAAA  
 5451 GCGTGTATGA AATGTTTCAGG AAGGATGACC CCCGCTAAG TTTCACACGC  
 5501 CAGTCCTTTG TGGACCGCTC CCTCCTCACT CTGCTGTGGC ACTGTAGCCT  
 5551 GGATGCTTTG AGAGAATTCT TCAGCACAAT TGTGGTGGAT GCCATTGATG  
 5601 TGTGAAGTC CAGGTTTACA AAGCTAAATG AATCTACCTT TGATACTCAA  
 5651 ATCACCAAGA AGATGGGCTA CTATAAGATT CTAGACGTGA TGTATTCTCG  
 5701 CCTTCCCAA GATGATGTTT ATGCTAAGGA ATCAAAAATT AATCAAGTTT  
 5751 TCCATGGCTC GTGTATTACA GAAGGAAATG AACTTACAAA GACATTGATT  
 5801 AAATTGTGCT ACGATGCATT TACAGAGAAC ATGGCAGGAG AGAATCAGCT  
 5851 GCTGGAGAGG AGAAGACTTT ACCATTGTGC AGCATACAAC TGCGCCATAT  
 5901 CTGTCATCTG CTGTGTCTTC AATGAGTTAA AATTTTACCA AGGTTTCTG  
 5951 TTTAGTGAAA AACCAGAAAA GAACTTGCTT ATTTTGA AAA ATCTGATCGA  
 6001 CCTGAAGCGC CGCTATAATT TTCTGTAGA AGTTGAGGTT CCTATGGAAA  
 6051 GAAAGAAAA GTACATTGAA ATTAGGAAAG AAGCCAGAGA AGCAGCAAAT  
 6101 GGGGATTCAG ATGGTCCTTC CTATATGTCT TCCCTGTCAT ATTTGGCAGA  
 6151 CAGTACCCTG AGTGAGGAAA TGAGTCAATT TGATTTCTCA ACOGGAGTTC  
 6201 AGAGCTATTG ATACAGCTCC CAAGACCCTA GACCTGCCAC TGGTCGTTTT  
 6251 CGGAGACGGG AGCAGCGGGA CCCCACGGTG CATGATGATG TGCTGGAGCT  
 6301 GGAGATGGAC GAGCTCAATC GGCATGAGTG CATGGCGCCC CTGAOGGCCC  
 6351 TGGTCAAGCA CATGCACAGA AGCCTGGGCC CGCCTCAAGG AGAAGAGGAT  
 6401 TCAGTGCCAA GAGATCTTCC TTCTTGATG AAATTCTCTC ATGGCAAAC  
 6451 GGGAAATCCA ATAGTACCAT TAAATATCCG TCTCTTCTTA GCCAAGCTTG  
 6501 TTATTAATAC AGAAGAGGTC TTTCGCCCTT ACGCGAAGCA CTGGCTTAGC  
 6551 CCCTTGCTGC AGCTGGCTGC TTCTGAAAAC AATGGAGGAG AAGGAATTCA  
 6601 CTACATGGTG GTTGAGATAG TGGCCACTAT TCTTTTCATG ACAGGCTTGG  
 6651 CCACTCCAAC AGGGGTCCCT AAAGATGAAG TGTTAGCAA TCGATTGCTT  
 6701 AATTTCTTAA TGAAACATGT CTTTCATCCA AAAAGAGCTG TGTTTAGACA



Figure 9b (vi)

6751 CAACCTTGAA ATTATAAAGA CCCTTGTCGA GTGCTGGAAG GATTGTTTAT  
6801 CCATCCCTTA TAGGTTAATA TTTGAAAAGT TTTCCGGTAA AGATCCTAAT  
6851 TCTAAAGACA ACTCAGTAGG GATTCAATTG CTAGGCATCG TGATGGCCAA  
6901 TGACCTGCCT CCCTATGACC CACAGTGTGG CATCCAGAGT AGCGAATACT  
6951 TCCAGGCTTT GGTGAATAAT ATGTCCTTTG TAAGATATAA AGAAGTGTAT  
7001 GCCGCTGCAG CAGAAATTCT AGGACTTATA CTTCGATATG TTATGGAGAG  
7051 AAAAAACATA CTGGAGGAGT CTCTGTGTGA ACTGGTTGCG AAACAATTGA  
7101 AGCAACATCA GAATACTATG GAGGACAAGT TTATTGTGTG CTTGAACAAA  
7151 GTGACCAAGA GCTTCCCTCC TCTTGACAGC AGGTTTCATGA ATGCTGTGTT  
7201 CTTTCTGCTG CCAAAATTTT ATGGAGTGTG GAAAACACTC TGTCTGGAGG  
7251 TGGTACTTTG TCGTGTGGAG GGAATGACAG AGCTGTACTT CCAGTTAAAG  
7301 AGCAAGGACT TCGTTCAAGT CATGAGACAT AGAGATGAAA GACAAAAAGT  
7351 ATGTTTGGAC ATAATTTATA AGATGATGCC AAAGTTAAAA CCAGTAGAAC  
7401 TCCGAGAACT TCTGAACCCC GTTGTGGAAT TCGTTTCCCA TCCTTCTACA  
7451 ACATGTAGGG AACAAATGTA TAATATTCTC ATGTGGATTG ATGATAATTA  
7501 CAGAGATCCA GAAAGTGAGA CAGATAATGA CTTCCAGGAA ATATTTAAGT  
7551 TGGCAAAAGA TGTGCTGATT CAAGGATTGA TCGATGAGAA CCTGGACTT  
7601 CAATTAATTA TTGAAAATTT CTGGAGCCAT GAAACTAGGT TACCTTCAAA  
7651 TACCTTGGAC CGGTGCTGG CACTAAATTC CTTATATTCT CTAAGATAG  
7701 AAGTGCACCT TTTAAGTTTA GCAACAAATT TTCTGCTCGA AATGACCAGC  
7751 ATGAGCCCAG ATTATCCAAA CCCCATGTTT GAGCATCCTC TGTCAGAATG  
7801 CGAATTTTCAG GAATATACCA TTGATTCTGA TTGGCGTTTC CGAAGTACTG  
7851 TTCTCACTCC GATGTTTGTG GAGACCAGG CCTCCAGGG CACTCTCCAG  
7901 ACCCGTACCC AGGAAGGGTC CCTCTCAGCT CGCTGGCCAG TGGCAGGGCA  
7951 GATAAGGGCC ACCCAGCAGC AGCATGACTT CACACTGACA CAGACTGCAG  
8001 ATGGAAGAAG CTCATTTGAT TGGCTGACCG GGAGCAGCAC TGACCCGCTG  
8051 GTCGACCACA CCAGTCCCTC ATCTGACTCC TTGCTGTTTG CCCACAAGAG  
8101 GAGTGAAAGG TTACAGAGAG CACCCTTGAA GTCAGTGGGG CTGATTTTGTG  
8151 GGAAAAAAG GCTGGGCCTT CCAGGGGACG AGGTGGATAA CAAAGTGAAA  
8201 GGTGCGGCCG GCCGACGGA CCTACTACGA CTGCGCAGAC GGTTTATGAG  
8251 GGACCAGGAG AAGCTCAGTT TGATGTATGC CAGAAAAGGC GTTGCTGAGC  
8301 AAAAACGAGA GAAGGAAATC AAGAGTGAGT TAAAAATGAA GCAGGATGCC  
8351 CAGGTGTTT TGTACAGAAG CTACCGGCAC GGAGACCTTC CTGACATTCA



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Figure 9b (v11)

8401 GATCAAGCAC AGCAGCCTCA TCACCCCGTT ACAGGCCGTG GCCCAGAGGG  
8451 ACCCAATAAT TGCAAAACAG CTCTTTAGCA GCTTGTTTTT TGGAAATTTTG  
8501 AAAGAGATGG ATAAATTTAA GACACTGTCT GAAAAAACA ACATCACTCA  
8551 AAAGTTGCTT CAAGACTTCA ATCGTTTTCT TAATACCACC TTCTCTTTCT  
8601 TTCCACCCCT TGTCTCTTGT ATTCAGGACA TTAGCTGTCA GCACGCAGCC  
8651 CTGCTGAGCC TCGACCCAGC GGCTGTTAGC GCTGGTTGCC TGGCCAGCCT  
8701 ACAGCAGCCC GTGGGCATCC GCCTGCTAGA GGAGGCTCTG CTCGCCTGC  
8751 TGCCTGCTGA GCTGCCTGCC AAGCGAGTCC GTGGGAAGGC CCGCTCCCT  
8801 CCTGATGTCC TCAGATGGGT GGAGCTTGCT AAGCTGTATA GATCAATTTG  
8851 AGAATACGAC GTCTCCGTG GGATTTTTAC CAGTGAGATA GGAACAAAGC  
8901 AAATCACTCA GAGTGCATTA TTAGCAGAAG CCAGAAGTGA TTATCTTGAA  
8951 GCTGCTAAGC AGTATGATGA GGCTCTCAAT AAACAAGACT GGGTAGATGG  
9001 TGAGCCCA CA GAAGCCGAGA AGGATTTTTG GGAAGTTGCA TCCCTTGACT  
9051 GTTACAACCA CCTTGCTGAG TGGAAATCAC TTGAATACTG TTCTACAGCC  
9101 AGTATAGACA GTGAGAAACC CCCAGACCTA AATAAAATCT GGAGTGAACC  
9151 ATTTTATCAG GAAACATATC TACCTTACAT GATCCGCAGC AAGCTGAAGC  
9201 TGCTGCTCCA GGGAGAGGCT GACCAGTCCC TGCTGACATT TATTGACAAA  
9251 GCTATGCACG GGGAGCTCCA GAAGGCGATT CTAGAGCTTC ATTACAGTCA  
9301 AGAGCTGAGT CTGCTTTACC TCCTGCAAGA TGATGTTGAC AGAGCCAAAT  
9351 ATTACATTCA AAATGGCATT CAGAGTTTTA TGCAGAATTA TTCTAGTATT  
9401 GATGTCTCT TACACCAAAG TAGACTCACC AAATTGCAGT CTGTACAGGC  
9451 TTTAACAGAA ATTCAGGAGT TCATCAGCTT TATAAGCAAA CAAGGCAATT  
9501 TATCATCTCA AGTTCCCTTT AAGAGACTTC TGAACACCTG GACAAACAGA  
9551 TATCCAGATG CTAAAATGGA CCCAATGAAC ATCTGGGATG ACATCATCAC  
9601 AAATCGATGT TTCTTTCTCA GCAAAATAGA GGAGAAGCTT ACCCTCTTTC  
9651 CAGAAGATAA TAGTATGAAT GTGGATCAAG ATGGAGACCC CAGTGACAGG  
9701 ATGGAAGTGC AAGAGCAGGA AGAAGATATC AGCTCCCTGA TCAGGAGTTG  
9751 CAAGTTTTTC ATGAAAATGA AGATGATAGA CAGTGCCCGG AAGCAGAACA  
9801 ATTTCTCACT TGCTATGAAA CTACTGAAGG AGCTGCATAA AGAGTCAAAA  
9851 ACCAGAGACG ATTGGCTGGT GAGCTGGGTG CAGAGCTACT GCCGCCTGAG  
9901 CCACTGCCCG AGCCGGTCCC AGGGCTGCTC TGAGCAGGTG CTCACTGTGC  
9951 TGAAAACAGT CTCTTTGTTG GATGAGAACA ACGTGTCAAG CTACTTAARC  
10001 AAAAATATTC TGGCTTTCCG TGACCAGAAC ATTCTCTTGG GTACAACTTA  
10051 CAGGATCATA GCGAATGCTC TCAGCAGTGA GCCAGCCTGC CTTGCTGAAA



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Figure 9b (v111)

10101 TCGAGGAGGA CAAGGCTAGA AGAATCTTAG AGCTTTCTGG ATCCAGTTCA  
10151 GAGGATTTCAG AGAAGGTGAT CGCGGGTCTG TACCAGAGAG CATTCAGCA  
10201 CCTCTCTGAG GCTGTGCAGG CGGCTGAGGA GGAGGCCAG CCTCCCTCCT  
10251 GGAGCTGTGG GCCTGCAGCT GGGGTGATTG ATGCTTACAT GACGCTGGCA  
10301 GATTTCTGTG ACCAACAGCT GCGCAAGGAG GAAGAGAATG CATCAGTTAC  
10351 TGATTCTGCA GAACTGCAGG CGTATCCAGC ACTTGTTGGT GAGAAAATGT  
10401 TGAAAGCTTT AAAATTAAAT TCCAATGAAG CCAGATTGAA GTTTCCTAGA  
10451 TTACTTCAGA TTATAGAACG GTATCCAGAG GAGACTTTGA GCCTCATGAC  
10501 AAAAGAGATC TCTTCCGTTT CCTGCTGGCA GTTCATCAGC TGGATCAGCC  
10551 ACATGGTGGC CTTACTGGAC AAAGACCAAG CCGTTGCTGT TCAGCACTCT  
10601 GTGGAAGAAA TCACTGATAA CTACCCGAG GCTATGTGTT ATCCCTTCAT  
10651 CATAAGCAGC GAAAGCTATT CCTTCAAGGA TACTTCTACT GGTGATAAGA  
10701 ATAAGGAGTT TGTGGCAAGG ATTAAGAGTA AGTTGGATCA AGGAGGAGTG  
10751 ATTCAAGATT TTATTAATGC CTTAGATCAG CTCTCTAATC CTGAACTGCT  
10801 CTTTAAGGAT TGGAGCAATG ATGTAAGAGC TGAAGTAGCA AAAACCCCTG  
10851 TAAATAAAAA AAACATTGAA AAAATGTATG AAAGAATGTA TGCAGCCTTG  
10901 GGTGACCCAA AGGCTCCAGG CCTGGGGGCC TTTAGAAGGA AGTTTATTCA  
10951 GACTTTTGGG AAAGAATTTG ATAAACATTT TGGGAAAGGA GGTTCATAAC  
11001 TACTGAGAAT GAAGCTCAGT GACTTCAACG ACATTACCAA CATGCTACTT  
11051 TTAAAAATGA ACAAAGACTC AAAGCCCCCT GGAATCTGA AAGAATGTTT  
11101 ACCCTGGATG AGCGACTTCA AAGTGGAGTT CCTGAGAAAT GAGCTGGAGA  
11151 TTCCCGGTCA GTATGACGGT AGGGGAAAGC CATTGCCAGA GTACCACGTG  
11201 CGAATCGCCG GGTTTGATGA GCGGGTGACA GTCATGGCGT CTCTGCGAAG  
11251 GCCCAAGCGC ATCATCATCC GTGGCCATGA CGAGAGGGAA CACCCCTTTC  
11301 TGGTGAAGGG TGGCGAGGAC CTGCGGCAGG ACCAGCGCGT GGAGCAGCTC  
11351 TTCCAGGTCA TGAATGGGAT CCTGGCCCAA GACTCCGCCT GCAGCCAGAG  
11401 GGCCCTGCAG CTGAGGACCT ATAGCGTTGT GCCCATGACC TCCAGTGATC  
11451 CCAGGGCACC GCCGTGTGAA TATAAAGATT GGCTGACAAA AATGTCAGGA  
11501 AAACATGATG TTGGAGCTTA CATGCTAATG TATAAGGGCG CTAATCGTAC  
11551 TGAAACAGTC ACGTCTTTTA GAAAACGAGA AAGTAAAGTG CCTGCTGATC  
11601 TCTTAAAGCG GGCCTTCGTG AGGATGAGTA CAAGCCCTGA GGCTTTCTGT  
11651 GCGCTCCGCT CCCACTTCGC CAGCTCTCAC GCTCTGATAT GCATCAGCCA  
11701 CTGGATCCTC GGGATTGGAG ACAGACATCT GAACAACCTT ATGGTGGCCA



Figure 9b (ix)

11751 TGGAGACTGG CGGCGTGATC GGGATCGACT TTGGGCATGC GTTTGGATCC  
11801 GCTACACAGT TTCTGCCAGT CCCTGAGTTG ATGCCMTTTC GGCTAACTCG  
11851 CCAGTTTATC AATCTGATGT TACCAATGAA AGAAACGGGC CTTATGTACA  
11901 GCATCATGGT ACACGCACTC CGGGCCTTCC GCTCAGACCC TGGCCTGCTC  
11951 ACCAACACCA TGGATGTGTT TGTCAAGGAG CCCCTCTTTG ATTGGAAAAA  
12001 TTTTGAACAG AAAATGCTGA AAAAAGGAGG GTCATGGATT CAAGAAATAA  
12051 ATGTTGCTGA AAAAAATTGG TACCCCGAC AGAAAATATG TTACGCTAAG  
12101 AGAAAGTTAG CAGGTGCCAA TCCAGCAGTC ATTACTTGTG ATGAGCTACT  
12151 CCTGGGTCAT GAGAAGGCCC CTGCCTTCAG AGACTATGTG GCTGTGGCAC  
12201 GAGGAAGCAA AGATCACAAC ATTCTGTGCC AAGAACCAGA GAGTGGGCTT  
12251 TCAGAAGAGA CTCAAGTGAA GTGCCTGATG GACCAGGCAA CAGACCCCAA  
12301 CATCCTTGGC AGAACCTGGG AAGGATGGGA GCCCTGGATG TGAGGTCTGT  
12351 GGGAGTCTGC AGATAGAAAG CATTACATG TTTAAAGAAT CTACTATACT  
12401 TGGTTGGCAG CATTCCATGA GCTGATTTTC CTGAAACACT AAAGAGAAAT  
12451 GTCCTTTGTG CTACAGTTTC GTAGCATGAG TTTAAATCAA GATTATGATG  
12501 AGTAAATGTG TATGGGTAA ATCAAAGATA AGGTTATAGT AACATCAAAG  
12551 ATTAGGTGAG GTTTATAGAA AGATAGATAT CCAGGCTTAC CAAAGTATTA  
12601 AGTCAAGAAT ATAATATGTG ATCAGCTTTC AAAGCATTTA CAAGTGCTGC  
12651 AAGTTAGTGA AACAGCTGTC TCCGTAAATG GAGGAAATGT GGGGAAGCCT  
12701 TGGAATGCCC TTCTGGTTCT GGCACATTGG AAAGCACACT CAGAAGGCTT  
12751 CATCAACAAG ATTTTGGGAG AGTAAAGCTA



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Fig.10.

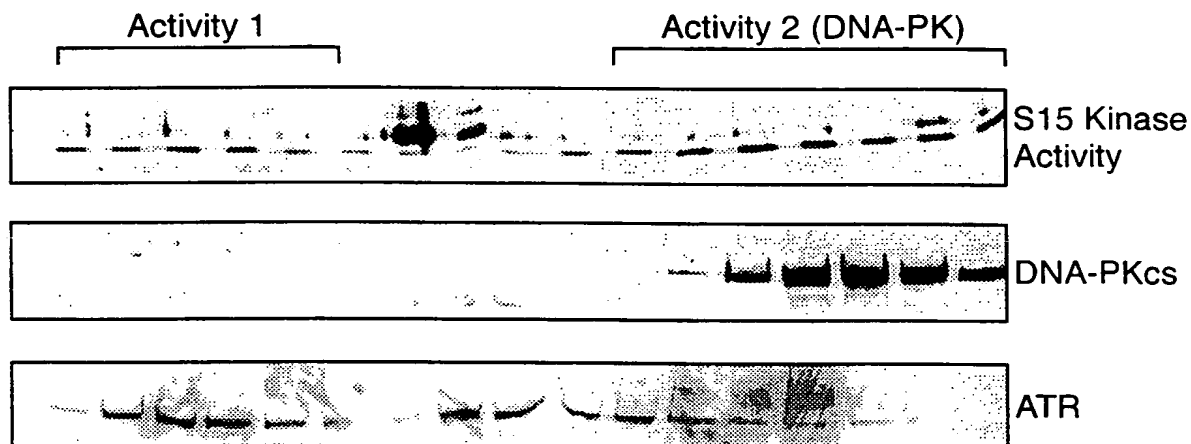


Fig.11.

